SPECIATION, VIRULENCE FACTORS DETECTION AND ANTIFUNGAL SUSCEPTIBILITY TESTING OF CANDIDA ISOLATED FROM HETEROGENOUS CLINICAL SAMPLES

Dissertation submitted in partial fulfillment of the

Requirement for the award of the Degree of

M.D. MICROBIOLOGY (BRANCH IV)

CHENNAI MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTRE

IRUNGALUR, TRICHY- 621 105

Affiliated To

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY,

CHENNAI, TAMILNADU

APRIL– 2017.

CERTIFICATE

CERTIFICATE

This is to certify that the dissertation entitled, **"Speciation, Virulence Factors Detection And Antifungal Susceptibility testing of Candida Isolated from Heterogenous Clinical Samples"** by **Dr.Shalini.M**, Post graduate in Microbiology (2014-2017), is a bonafide research work carried out under our direct supervision and guidance and is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, for M.D. Degree Examination in Microbiology, Branch IV, to be held in April 2017.

Guide:

Dr. A. Uma M.D,
Professor and Head,
Department of Microbiology,
CMCH&RC.

Professor and Head:

Dr. A. Uma M.D,
Professor and Head,
Department of Microbiology,
CMCH&RC.

Dean:

Dr. Sukumaran Annamalai M.D, D.H.H.M.,
Chennai Medical College Hospital and Research Centre,
Irungalur,
Thiruchirapalli-621 105.
Tamil Nadu.

DECLARATION

I solemnly declare that the dissertation titled **"Speciation, Virulence Factors Detection and Antifungal Susceptibility testing of Candida isolated from Heterogenous Clinical Samples"** is bonafide record of work done by me at Chennai Medical College Hospital and Research centre, Trichy.

The dissertation is submitted to The Tamil Nadu Dr.M.G.R Medical University towards the partial fulfillment of requirements for the award of M.D Degree (Branch IV) in Microbiology.

Place: Trichy

Date**:**

Dr. Shalini.M
Post Graduate Student,
M.D Microbiology,
Chennai Medical College Hospital and Research Centre
Irungalur,
Trichy.

CHENNAI MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE

IRUNGALUR, TRICHY – 621 105.
E.Mail : iec.cmchrc@gmail.com, Phone: 0431-3058661,3058817

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1.0 Introduction
Candida species are ubiquitous yeast like fungi associated with human beings for a quite long time. There are about 200 species of Candida, dwelling as saprophytes in soil and aquatic environment and also colonizing several animal reservoirs. In about 70% of healthy individuals, Candida exists as commensals of the gastrointestinal and genitourinary tracts [1].
Whenever there is alteration in the equilibrium between Candida and the host factors, the Candida which is a commensal becomes pathogenic and causes several diseases. Under such situations Candida species causes superficial, invasive or disseminated infection by infecting all the sites of the human body. The infections
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ACKNOWLEDGEMENT

I humbly submit this work to **ALMIGHTY**, who has given me the strength, endurance and ability to overcome the difficulties encountered in the process of compilation of my dissertation work.

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Abbreviations

C.albicans	*Candida albicans*
C.tropicalis	*Candida tropicals*
C.parapsilosis	*Candida parapsilosis*
C.krusei	*Candida krusei*
C.glabrata	*Candida glabrata*
GTT	Germ Tube Test
SDA	Sabourauds Dextrose Agar
UTI	Urinary Tract Infection
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
VVC	Vulvo Vaginal Candidiasis
SAP	Secreted Aspartyl Proteinase Enzyme
IDSA	Infectious Disease Society of America
CDC	Centre For Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ELISA	Enzyme Linked Immune Sorbent Assay
HSP	Heat Shock Proteins
GM -CSF	Granulocyte Macrophage Colony Stimulating Factor
M- CSF	Macrophage Colony Stimulating Factor
G - CSF	Granulocyte Colony Stimulating Factor

INTRODUCTION

1.0 INTRODUCTION

Candida species are ubiquitous yeast like fungi associated with human beings for a quite long time. There are about 200 species of *Candida*, dwelling as saprophytes in soil and aquatic environment and also colonizing several animal reservoirs. In about 70% of healthy individuals, *Candida* exists as commensals of the gastrointestinal and genitourinary tracts [1].

Whenever there is alteration in the equilibrium between *Candida* and the host factors, the *Candida* which is a commensal becomes pathogenic and causes several diseases. Under such situations *Candida* species causes superficial, invasive or disseminated infection by infecting all the sites of the human body. The infections includes oral thrush, glossitis, vulvovaginitis, intertrigo, paronychia, urinary tract infection, endocarditis to meningitis [2].

The predisposing factors for candidiasis includes [3]

- Prolonged administration of antibiotics
- Immuno- compromised states such as
    - Acquired immuno deficiency syndrome,
    - Cancer chemotherapy,
    - Immunosuppressants,
    - Diabetes mellitus,
    - Extremes of age,
    - Burns,
    - Steroids,
    - Pregnancy,

- Prolonged hospital stay,
- Genetic deficiency syndromes,
- Prolonged Antibiotic therapy,
- Insertion of catheters and unsterile needles.

Candida has emerged as a major cause of human disease and the fungal infection rates have been increasing over the past 20 years [4].

Candidiasis has worldwide distribution. It is the commonest cause of hospital acquired blood stream infections in United States [5].

In HIV patients, candidiasis is the second most common infection [6]. The rate of invasive candidiasis have increased with increase in the epidemic of HIV. 80% of AIDS patients developed oral candidiasis before the advent of Highly Active Anti Retroviral Therapy regimen. Oro pharyngeal candidiasis is most common among the HIV patients and it is the important marker for immunosuppression [2].

Candidiasis was found to be the second most common infection in patients on cancer chemotherapy and transplant recipients [7].

Candida causing nosocomial UTI has become most prevalent with an increased mortality of about 10- 15% [8].

Eighty percent of women suffer from vulvovaginal candidiasis (VVC) atleast once in their life time, although all the other organs are also frequently infected with Candidiasis[3].

Candida albicans accounts for 40-60% yeasts isolated in developed countries, whereas Indian reports show an increased occurrence of infection with *Candida non albicans* species. *Candida tropicalis* has the highest adherence rate to

inanimate materials such as urinary and vascular catheters, and is often involved in biofilm formation.

From 1970 to 2000 *Candida albicans* dominated as causal *Candida* pathogen worldwide in all forms of candidal infections. Significant changes has occurred in the last decade have transpired with the progressive important role of *Candida non albicans* species imparting a profound influence on selection of antifungal drugs [9].

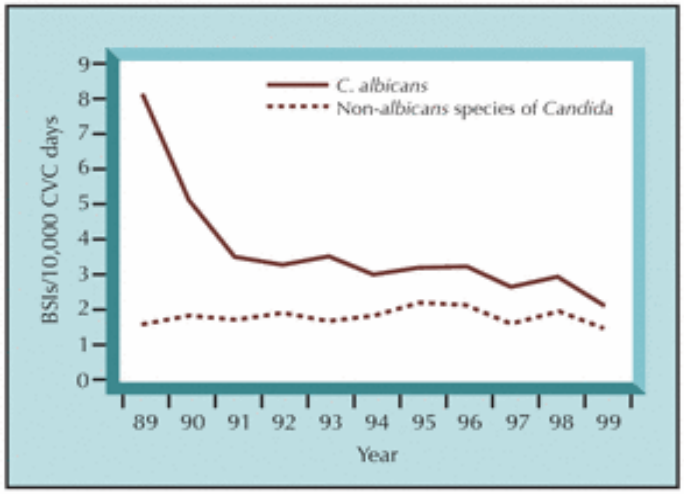


Fig 1: Emergence of *Candida* non albicans

Source: http://link.springer.com/article/10.1007

The extent and severity of these candidial infections depends on the immune status of the host. The *Candida* species exhibits certain virulence factors that helps the organism in proliferation, adhesion and invasion of host tissue. The extracellular hydrolytic enzymes produced by *Candida* species helps in proliferation, prevents the phagocytosis and helps in the survival of the organism. Biofilm formation plays an important role in the pathogenesis. Different species of *Candida* exhibits different virulence factors for their survival [10].

Infectious diseases society of America (IDSA) guidelines 2016 states that – candidiasis is a serious, life threatening infection that needs to be treated early, aggressively and appropriately.

There are various antifungals used in the treatment of candidiasis. These consist of fluconazole, voriconazole, caspofungin, amphotericin B, and lipid formulations of amphotericin B. Fluconazole is most commonly used in the treatment of candidemia.

Resistance to antifungal drugs is increasing in recent years [11]. The resistance of *C.albicans, C.tropicalis and C.glabrata* to fluconazole is increased compared to other drugs, due to indiscriminate use of fluconazole for long periods [12]. *C.krusei* is intrinsically resistant to fluconazole. IDSA also states that, more than 90 % of invasive candidiasis is caused by *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis,* and *C. krusei*, and these have unique virulence potential, antifungal susceptibility and epidemiology[13]. All these factors has led to increase in the mortality and morbidity rates in patients with fungal infections warranting rapid identification and antifungal susceptibility testing at the earliest.

Therefore due to the variable clinical presentation of *Candida* infections, it has become important to identify the *Candida* species from all the clinical specimens. Differentiating among *Candida* species in laboratory is also very important because of the differences in the virulence of the species and in their susceptibility to anti-fungal drugs.

Hence the present study was conducted in a tertiary care hospital with 770 beds and an outpatient turnover of 1330 per day, to isolate the *Candida* from

heterogenous clinical samples, speciate it. The virulence factors and the drug susceptibility pattern was studied for the different species for appropriate and effective treatment.

AIMS AND OBJECTIVES

2.0 AIMS OF THE STUDY

- To isolate and identify *Candida* from heterogenous clinical samples in a tertiary care hospital.
- To speciate and evaluate their distribution with the age, clinical diagnosis and co-morbid conditions.
- To differentiate between commensal and pathogenic *Candida* species by determining the virulence factors of the isolated *Candida* species.
- To determine the in vitro efficacy of antifungal agents of *Candida* species.
- To determine the association of virulence factors with age, co-morbid conditions and their antifungal resistance pattern.

REVIEW OF LITERATURE

3.0. REVIEW OF LITERATURE

3.1. History:

Candidiasis has a very old history, the disease was described in ancient times. Hippocrates around 400 B.C. had described oral candidiasis as "mouth affected with aphthous ulcerations" in "Of the Epidemics"[14].

Table:1 History of *Candida* in chronological order

Year	Author	Event
1665	Pepy's	Description of the disease was noted in his diary as described by Galen [2]
1751	Hill	Isolated the yeast from rotting vegetation and was confused with the term "Monillia" which is often confused with *Candida* [15]. But the organism isolated was an Aspergillus species.
1771	Rosen Von Rosenstein	Defined the invasive form of thrush [16].
1784	Underwood	Described oral and gastrointestinal candidiasis as a disease of pediatric age group [17]
1844	Bennet	Isolated the yeast from the sputum of tuberculosis patient [18]
1847	Charles Philippe Robin	French mycologist, named the fungus causing thrush as *Oidium albicans* ("to whiten").
1861	Zenker	Isolated from brain of a debilitated patient in whom the yeast spread from his oral thrush by blood [11]
1874	Parrot	Noted the first pulmonary infection case caused by *Candida*
1894		*Torulopsis glabrata* was isolated from grapes
1954		The name *Candida* was officially accepted by the 8th Botanical Congress held at Paris in [2]. The word *Candida* is derived from Latin, where toga

		Candida was a white robe worn by Roman Senators [19].
1959	Viswanathan & Randhawa	Isolated *Candida viswanathi,* named in honour of Dr.R.Viswanathan, the first director of the Vallabhai Patel Chest Institute Delhi [2].
1959	Galen	Described candidiasis as the disease commonly seen in sick children[20].
1978		*Torulopsis glabrata* was merged into this genus and named as name *Candida glabrata* [2].
1995	Sullivan and colleagues	From Dublin isolated a new species, *Candida dubliniensis* [21]

Thus, with the advent of antimicrobial agents in the latter half of the twentieth century and increase in the immunosuppressed patients in the last few decades have restored the interest in *Candida* and candidiasis. These events have led to an increase of *Candida* infection particularly the less pathogenic *non albicans* species [22].

3.2 Taxonomy [12,23]:

Candida falls under :

Table 2 : Taxonomic hierarchy of *Candida*	
Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Ascomycotina
Class	Ascomycetes
Order	Saccharomycetales
Family	Sacharomycetaceae
Genus	*Candida*

The genus *Candida* consists of approximately 200 species out of which 20 have been associated with pathology to man [2,24]

The major pathogenic species include the following:

Table 3:Major pathogenic species of *Candida*	
• *Candida albicans* • *Candida dubliniensis* • *Candida tropicalis* • *Candida glabrata* • *Candida guilliermondii*	• *Candida kefyr* • *Candida krusei* • *Candida lusitaniae* • *Candida parapsilosis*

Candida tropicalis is the most common *Candida* species isolated in patients with hematologic malignancies and bone marrow transplant recipients [25].

Candida parapsilopsis is frequently isolated from pathological lesion of the nails and skin. It is reported as the causative agent in endocarditis, endophthalmitis, septic arthritis and peritonitis.

Candida glabrata is relatively a non pathogenic and normal flora of healthy individuals. But following the wide spread and increased use of immunosuppressives together with broad spectrum antifungal therapy, the frequency of mucosal and systemic infections caused by *Candida glabrata* among the old age have been increased [25].

The prevalence of *Candida albicans* and *Candida non albicans* has changed. The prevalence of *C.glabrata* increases with age and common among patients aged ≥ 70 years, whereas in *C.parapsilosis* it is the other way [26].

In a study, done by Kothari et al. *C.tropicalis* causes 45% of infections, *C.albicans* 23% and other species of *Candida* 23% [27].

A study done by Chakrabarti et al reported that *Candida non albicans* is more prevalent (86.4%) than *C.albicans* (11.4%)[28].

3.3. Physiology :

Candida species metabolise glucose via the Hexose Monophosphate pathway under aerobic conditions (assimilation) or via the Embden Meyerhof pathway in anaerobiosis (fermentation). Mitochondrial oxidative phosphorylation, Kreb's cycle and protein synthesis are similar to those of eukaryotic cells. *Candida* enzymes are significant as they may be directly involved in pathogenesis. Growth temperature has an important influence on morphogenesis. Temperatures around 25°C promote the formation of chlamydospores in *C.albicans* and higher temperatures around 37°C promote formation of pseudohyphae [12].

3.4. Epidemiology

Fungi are ubiquitous in plants, mammals and insects. Humans are continually exposed to numerous genera of fungi through a variety of routes. *Candida* are commensals of humans and are commonly found on skin, gastrointestinal tract and genito urinary tract[29].

In United States, the colonization of *Candida albicans* in the oropharynx can be found in almost 30-55% of young adults [30]. Also, the presence of *C. albicans* in normal fecal sample is about 40-65% [30]. Candidemia is the fourth most common cause of bloodstream infection, Almost 6.9 out of every 1000 intensive care unit patients are suffering from candidemia [31].

C. albicans was found to be the most frequently isolated pathogen in Northern and Central Europe and the USA, whereas *Candida non albicans* species were found to predominate in Asia, Southern Europe and South America[32]

The highest proportion of *C.glabrata* isolates were found in Northern and Central Europe, whereas *C.parapsilosis* was most commonly found in Slovakia, Southern Europe, South America and Asia and *C.tropicalis* predominated in Eastern Asia and Argentina[32].

Nosocomial transmission or 'cross-infection' of Candidiasis poses significant problems. Recent literatures have reported the isolation of *Candida* from the hands of health care workers in intensive care units and cross- infection. Epidemiologic studies showed that *Candida non albicans* are more frequently isolated from the urine samples than the other clinical samples, this may be probably due to urine composition and or pH. Mixed isolates were found in patients with nosocomial Candiduria. The emergence of *Candida non albicans* species may represent selection of more resistant species like *C. glabrata* and *C.krusei*[33].

3.5. Pathology and pathogenesis:

Candida is a commensal in different site of the body. So the source is mainly endogenous[34] and rarely can be introduced exogenously [35]. The exogenous source includes insertion of catheters or prosethetic valves [36]. The repeated isolation from the same patient and in significant number indicates infection or colonization. But any isolation from sterile body fluids is significant. In immunocompetent individuals immune system combats the infection.

3.5.1. Immunity:[2,37].

- Classical T- cell immunity → protection from persistent high level mucocutaneous *Candida* colonization

- Polymorpho nuclear cells → damages and phagocytoses the pseudohyphae → protects from deep seated candidiasis
- Cytokines (INF-α, IL-3, GM-CSF, M-CSF, G-CSF) → phagocytoses the yeast.
- Serum factors, and heat labile opsonins → increased ingestion of yeast; thereby B-lymphocytes and antibodies also have a role.

3.5.2. Virulence factors:

There are many virulence factors which plays important role at different sites and stages of infection. Virulence factors characterized in terms of function and not considering the antigenicity[2].

Virulence factors are [2]:

- Adhesion
- Enzymes
- Toxins
- Complement receptors
- Phenotype switching

3.5.2. a) Adhesion:

Ability of C*andida* to adhere to the host cell correlates with their virulence and plays a major role in pathogenesis. Adhesion present on the surface of fungus interacts with the receptor of the host cell thereby helps in binding with the epithelial and the endothelial cells. *Candida* has an ability to bind with the host cells both in vivo and in vitro, tried in experimental animals [38]. In vitro *Candida* is capable of adhering to exfoliated human epithelial cells, human tissue, cell lines (endothelial, Hela) and inert surfaces like various polymers used as indwelling medical devices[12].

3.5.2 .b) Enzymes:

There are about 14 hydrolytic enzymes but only two plays a role in pathogenesis. They include proteases, lipases, phospholipases, esterases, phosphatases etc. Secretion of secreted aspartyl proteinase enzyme (Sap) plays a major role in pathogenesis[39]. Sap was produced by pathogenic *Candida* species after adhesions to epithelial cells[40]. There are three types of Sap and two types of serine proteinases which are involved in growth control of yeast remodeling and they also facilitate hyphal invasion leading to disseminated candidiasis [2,41]. Mutants which are deficient in Sap are unable to invade the host tissue[42]. Phospholipase enzyme is secreted during the tissue penetration and is essential for the virulence[39].

3.5.2. c) Toxins:

The glycoprotein extracts of *Candida* cell wall resembles endotoxins of bacteria. These are lethal and pyrogenic → anaphylactic shock.

3.5.2. d) Complement receptors:

It binds to the complement derived opsonins → plays a role in virulence.

3.5.2. e) Phenotypic Switching:

It is the ability of a single strain to switch reversibly at high frequencies among different colony phenotypes. This evades the host immune response.

3.5.3. *Candida* Antigens:

The antigens of *Candida* is divided into 2 main groups:

i. Cell wall antigens

ii. Cytoplasmic antigens

3.5.3 i) Cell wall antigens :

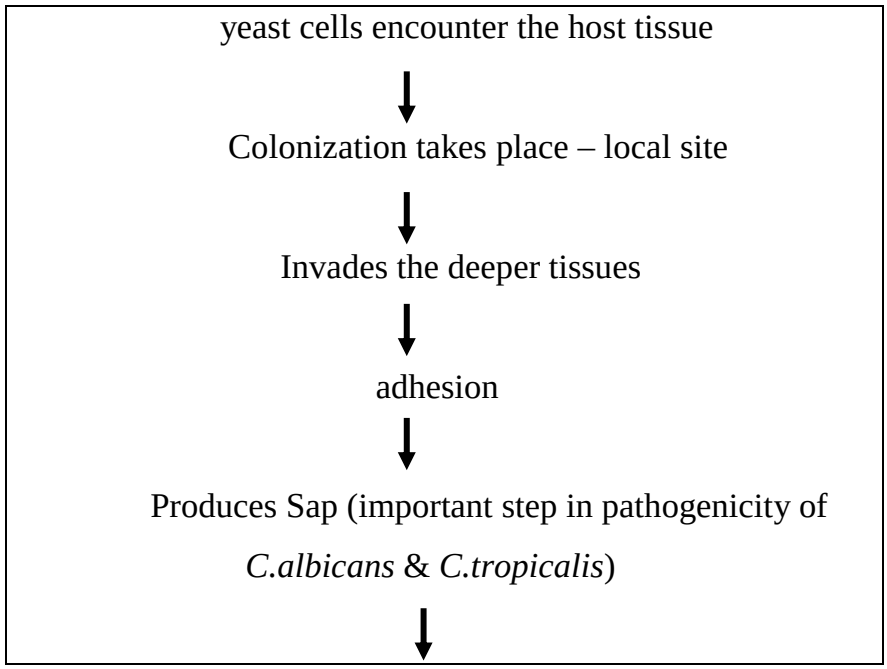
Fungal surface molecules include mannan, glucans and chitin contributes to the virulence of *Candida* species. It causes suppression of immune response and adherence of the host tissue by changing the hydrophobicity [43]. The mannoproteins released from *Candida* albicans binds with the red blood cells therby causing hemolysis of red cells. These hyphal cells uses hemoglobin as iron source [44].

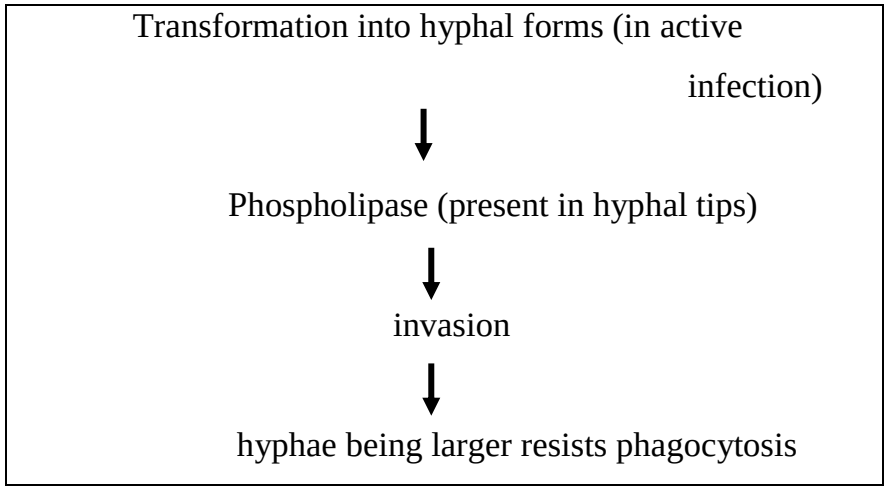
These organisms attaches and grows in colonies and causes biofilm formation[45]. The biofilm contains extracellular materials like proteins, carbohydrates etc [46]. The antimicrobials have poor penetration in biofilms [47].

3.5.3 ii) Cytoplasmic antigens:

A number of antigenic components in extracts of broken cells are also considerably significant. These antigens have not yet been associated with specific cytoplasmic components of the cell.

3.5.4. Virulence factors contributing to Pathogenesis:





Flow chart showing pathogenesis

The pathogenicity of *Candida* depends on complex array of micro organism related putative virulence factors. These include[2]

- Yeast to mycelium transition
- Antigenic variability
- Phenotypic switching
- Adhesion to host cells and tissue
- Cell surface hydrophobicity
- Molecular mimicry
- Production of extracellular enzymes

Most of the biological functions related to pathogenicity and virulence are confined to the cell wall [2].

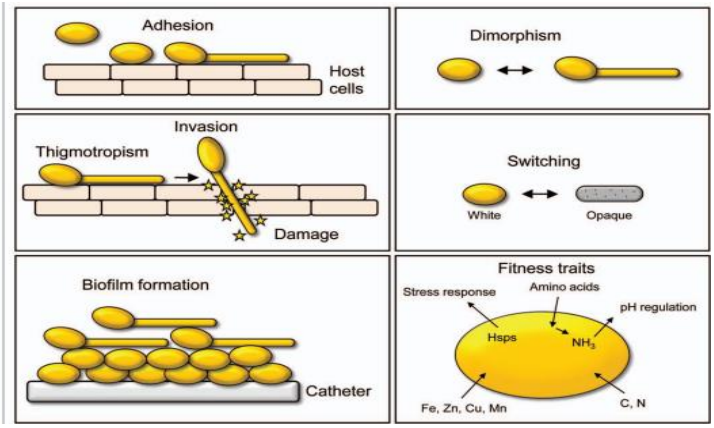


Fig 2 : Pathogenic mechanism

Source: Virulence. 2013 Feb 15; 4(2): 119–128.

3.6 .Clinical features:

The clinical manifestation of *Candida* is varied, ranging from acute, subacute, chronic and episodic[2]. Involvement may be localized or systemic. The pathologic process vary from irritation and inflammation to chronic and acute suppuration or granulomatous response [2].

Clinical classification of candidiasis [2]:

Table 4: Clinical classification of candidiasis

I) Infectious disease

A) Mucocutaneous manifestation

- Oral[48,49,50] : thrush, stomatitis, glosssitis, cheilitis
- Alimentary [51]: esophagitis, gastritis.
- Vulvovaginitis[52], balanitis, balanoposthitis.
- Chronic mucocutaneous candidiasis[12]
- Ocular candidiasis [53]

B) Cutaneous manifestation

- Intertriginous [54]and generalized
- Paranychia and onchomycosis[55]

• Diaper dermatitis[2] • Candidial granuloma C) Systemic manifestations • Urinary tract – candiduria[56,57] • Endocarditis [58,59] • Pulmonary candidiasis • Meningitis [35] • Candidemia • Dissemination • Arthritis and osteoarticular candidiasis • Osteomyelitis • Endophthalmitis [61] • Invasive candidiasis
II) Allergic diseases • Candidids • Eczema • Asthma • Gastritis

3.6.1. Invasive candidiasis and candidemia:

Invasive candidiasis is a multiorgan infection. *Candida* is not isolated from blood culture [12]. It is common among acute leukaemia patients, post operative patients, cancer patients, transplant patients, prolonged ICU admission and also in drug addicts [12]. Patients with candidemia presents with nodular lesions of the skin and white lesions of the retina. These signs are important in the diagnosis even when the culture is negative for pathogen[35].

Risk factors for the development of invasive candidiasis [60].

Table 5 : Risk factors for invasive candidiasis
• Hematological or solid malignancies, • Neutropenia • Renal failure • Severe acute pancreatitis • Organ transplantation • Long hospitalization period in the intensive care unit • High APACHE II (Acute Physiology and Chronic Health Evaluation) score • Hemodialysis • Usage of antibiotics with broad spectrum • Usage of antifungal agents • Presence of central venous catheters • Mechanical ventilation • Total parenteral nutrition • Usage of immunosuppressive agents For neonates and children, in addition to adults • Prematurity • Low birth weight • Low APGAR (American Pediatric Gross Assessment) score • Congenital malformations

3.6.2. Clinical forms of candidiasis in HIV patients [2]:

Table 6 :Clinical features of candidiasis in HIV
• Asymptomatic oral carriage • Oropharyngeal thrush • Acute atrophic erythema

- Perleche (angular cheilitis)
- Leukoplakia
- Oesophagitis
- Laryngitis
- Vulvovaginitis, balanitis
- Hematogenous dissemination

3.6.3.Fungal infections in ICU:

Invasive candidiasis is more common in immunosuppressed condition. But recent studies have shown that invasive candidiasis can occur even in immunocompetent patients who are critically ill[62]. They have non specific clinical presentation and high mortality rates. The risk factors associated with invasive candidiasis are compromised immune function either due to debilitating clinical condition or use of immune suppressing medications and also prolonged stay in Intensive care units.

Other risk factors include [62]:

- *Candida* colonization at various body sites
- Broad spectrum antibiotics
- Immunosuppressive therapy like cytotoxic chemotherapy
- Corticosteroids
- Malnutrition
- Malignancy
- Neutropenia
- Severe burns and
- Indwelling catheter.

3.7. Diagnosis of candidial infection :

There are nearly 20 species of *Candida* which are pathogenic to humans. The difficulty in the diagnosis lies due to the absence of specific symptoms and signs as well as the opportunistic nature of the yeast. *Candida* species identification plays a major role in successful management.

3.7.1 Specimen:

Specimens are collected depending upon the site of infection. Sputum, bronchial aspirate, exudates, scrapings from mucosal, dermal or nail lesions etc. are collected.

3.7.2 A) Direct examination:

The samples are examined in KOH wet mount or normal saline preparation for the visualization of yeast cells and pseudohyphae.

Gram staining is performed for the presence Gram positive budding yeast cells and pseudohyphae. The yeast cells are approximately 4-8µm. They are non-capsulated. The pseudohyphae show regular point of constriction. The microscopy indicates only candidiasis and the etiology can be established only by culture[12]. The pseudohyphae shows colonization and tissue invasion, hence their demonstration indicates clinical importance.

Calcofluor white stains are used to highlight the fungal elements. Other stains used are H&E and Gomori's methenamine silver stain.

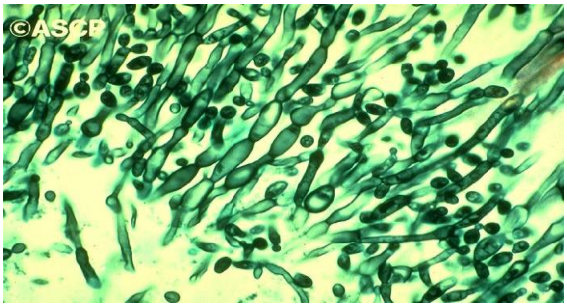


Fig 3: Methenamine silver stain- *Candida albicans*

Source: http://www.microbiologybook.org/mycology

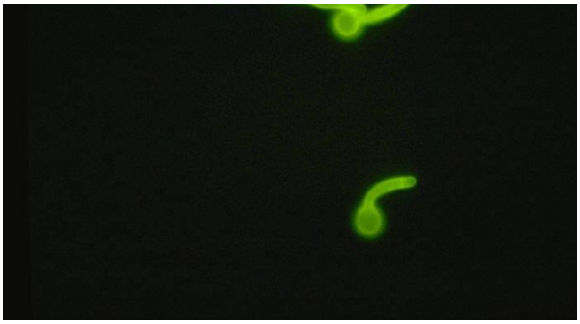


Fig 4: Calcofluor white stain - *Candida albicans*

Source:http://www.microbiologybook.org/mycology

The biopsy specimen are kept in the tube containing 10% KOH for an overnight period at 37^0C and after mincing they are examined under microscope for yeast cells and pseudohyphae[2].

Table 7: Macroscopic and microscopic identification of *Candida* species		
Species	**Macroscopy**	**Microscopy**
C.albicans	Creamy smooth colonies. In old stocks it becomes waxy, soft, smooth sometimes reticulated, old cultures are wrinkled and folded with spicules[3].	Yeast cells are short and ovoid (5-7µm). Sometimes elongated yeasts which are smaller and larger cells are also seen [3].
C.tropicalis	**C**reamy white, smooth colonies. Older colonies- white to cream colored, dull soft and wrinkled, often	Yeast cells are ovoid or short ovoid cells (4-8x5-11µm) [3].

	with overgrowth of mycelium [3].	
C.glabrata	Smooth, soft, glossy and cream colored colony [3].	Yeast cells are smaller round to oval yeasts 2.5-to 4.5x 4-6μm [3].
C.parapsilosis	Younger colonies are smooth, soft sometimes lacy. Older colonies are creamy yellowish, glistening smooth or sometimes wrinkled [3]	Yeast cells are short ovoid to long ovoid (3-5x6-20μm) [3].
C.krusei	Colonies are flat, dull and dry and older colonies are greenish yellow, dull, wrinkled with heavy growth of mycelium around the colonies [3].	Yeast cells are cylindrical, some of them ovoid to long (3-5x620μm) [3].
C.guilliermondii	Colonies are thin, flat, glossy, cream to pink colour. In old stocks colonies become yellowish cream to pink, glistening dull or smooth and wrinkled [3].	Yeast cells are ovoid and cylindrical (2-4x 3-7 μm) [3].
C.kefyr	Colonies are cream, smooth colony. In old cultures cream to yellow dull, soft and smooth [3].	Yeast cells are mostly small ovoid with few elongated cells (2.5 to 5 X 5 to 10μm) [3].
C.viswanathi	Younger colonies are cream-colored, soft, glistening; older colonies are creamy soft to membranous, wrinkled and dull [3].	Yeast cells are globose ovoid to cylindrical (2.5-7x4-1μm)[3].
C.lusitaniae	Cream-colored, soft, glistening [3].	Yeast Cells are ovoid (2 to 6 x 2.5 to 10μm) in pairs or chains. On Potato agar it forms Pseudomycelium with chains of blastoconidia [3].

3.7.2.B)Fungal culture:

Candida grows well in bacteriological basal or simple media like

nutrient agar and blood agar. The routinely used mycological media for *Candida* isolation is Sabouraud's dextrose agar (SDA) with added antibiotics (chloramphenicol or gentamycin) with pH adjusted to 5.6 [63]. SDA with cycloheximide should be avoided as it prevents the growth of *C.krusei*, *C.parapsilosis* and *C.tropicalis*. Most pathogenic species of *Candida* grows at 25 and 37°C [63]. *C.albicans* can also grow at 45°C.

The LPCB can be performed from the colonies for the presence of budding yeast cells and pseudohyphae. Gram staining also can be performed from the culture colonies.

For the detection of systemic Candidiasis blood culture can be done in biphasic medium like brain heart infusion agar – broth and incubated at both 25^0C and 37^0C. The colonies will be apparent by 24 hours or within 2- 3 days. The colony morphology of *Candida* species is smooth and creamy with fine differences. Some species produce dry colonies.

The growth of the *Candida* species is also seen on Tetrazolium Reduction Medium (TRM) and are compared with the standard colours.

3.7.2.C) Germ tube production [63]:

A germ tube is a filamentous extension from a parent cell. The true germ tube has no constriction at the neck. *C.albicans* and *C.dubliniensis* produce germ tubes when incubated with various substances like human or sheep serum, rabbit plasma, egg albumin, tissue culture medium, thioglycolate soya broth etc. at 37^0C for 2 hours. Not all strains of *C.albicans* produce germ tube. The demonstration of germ tube is known as Reynolds Braude phenomenon [64].

3.7.2. D) Corn meal agar inoculation

The commonly used differential media for identification of *Candida* upto species level. Speciation is done by the presence and position of chlamydospores, blastospores, hyphae and pseudohyphae.

3.7.2.E) *Candida* CHROM agar[63]:

It is a rapid, plate based test for the isolation and identification of species. Principle of the test is that different colour is formed due to the reaction between the enzymes in the organism and the chromogenic substrate in the medium.

Table :8 Features that differentiate *C.albicans* and *C.dubliniensis*

Characteristics	***C.albicans***	***C.dubliniensis***
Germ tube	+	++
Chlamydospores	+	+++
Growth on CHROM agar	**Light green**	**Dark green**
Growth at 42- 45^0 C	+	-

3.7.2.F) Biochemical tests:

Candida species are characterized by the pattern by which they use specific carbohydrate and nitrogen substances. The process by which the *Candida* species oxidatively utilize the carbohydrates is called as assimilation and anaerobical utilization of the carbohydrates is called as fermentation. If given yeast possesses the ability to ferment a carbohydrate it can also assimilate it.

1) Sugar fermentation tests:

This test involves peptone water added with various carbohydrates, a colour indicator and an inverted Durham tube. Colour indicator assesses the pH changes and measures acid production while Durham's tube detects gas production.

2) Sugar Assimilation tests:

Based on the ability of the *Candida* to utilize a specific carbohydrate in the presence of oxygen helps in species identification. The different techniques for detecting assimilation are as follows:

a. **Classical Wickerham and Bruton method** [65]

b. **Auxonographic technique** [63]

3.7.2.G) Commercial Yeast identification systems:

- API 20C system
- API ID32C
- API *Candida* kit
- VITEK (Biomerieux Vitek, Inc.,Hazelwood,MD)
- Minitek (Becton Dickinson Microbiology System,Cockeysville,MD)
- Uni-Yeast-1ck (Flow laboratories, Woodcock,UK).

3.7.2.H) Immunodiagnosis:

Antibody against *C.albicans* molecules or *Candida* derived molecules whose presence in sera indicates tissue invasion are identified.

a) Detection of Antibody [12]

- Gel immunodiffusion
- Counter immune electrophoresis
- ELISA and Latex agglutination tests.

b) Detection of Antigens [66]

- Mannan detection by ELISA

- Latex Agglutination systems using monoclonal and polyclonal antibodies.
- Glycoprotein detection using latex agglutination test.

c) Cell mediated immunity can be assessed by:

- Skin tests to detect delayed hypersensitivity to candidal antigen.
- In vitro assay like lymphocyte transformation test.

3.7.2.I) Molecular techniques

i) Detection of Candidial DNA by PCR using specific probes

ii) 26S rRNA for identification of yeast from blood culture bottles. It detects the organism within 2-3 hrs [67].

iii) Restriction fragment length polymorphism

iv) Southern hybridization pattern

3.7.2.J) MALDI-TOF:

It is a proteinomics used for rapid identification of *Candida* species. It is a powerful method for detection and identification of proteins by molecular weight of individual specific fragments[68].

3.7.3. *Candida* score:

It helps in early diagnosis of invasive candidiasis and helps the clinician to start early antifungal treatment [69]. It is calculated at the onset of sepsis or shock using total paraenteral nutrition, multifocal colonization, surgery and severe sepsis.

3.8. Treatment:

3.8.1. Evolution of antifungals[70]:

The fungal infections were known to exists for centuries, but the antifungal was

discovered only in 1939.

Table 9: Evolution of antifungals in chronological order		
Year	**Discoverer**	**Discovery**
1939	**Oxford et al**	**Griseofulvin was isolated and discovered from Penicillium griseofulvum**
1944	**Woolley et al**	**Azoles**
1958		**Griseofulvin used for clinical purpose**
1958		**Azoles was used as antifungal drug**
1957	**Duschinsky et al**	**5-Flurocytosine was used as anti tumor drug**
1960		**Clotrimazole, econazole, micanozole**
1963	**Greenberg & co workers**	**5-Flurocytosine was used as antifungal drug**
1968		**Micanozole was used for parenteral injection**
1981	**Heeres & co workers**	**Ketoconazole**
1992		**Itraconazole**
2001		**Itraconazoles – parenteral forms**
2002		**Voriconazole**
2006		**Posaconazole**
2001		**Caspofungin**
2005		**Micafungin**
2006		**Anidulafungin**

3.8.2.Antifungal agents and their Mechanism of action [71]:

Table 10. Antifungals and their mechanism of action		
Chemical class	**Drug**	**Target**
Azoles	**Miconazole** **Ketoconazole** **Fluconazole** **Itraconazole** **Teroconazole**	**Ergosterol synthesis**

	Voriconazole **Posaconazoles**	
Polyenes	**Amphotericin B** **Nystatin**	**Ergosterol membrane function**
Pyrimidine	**Flucytosine**	**DNA & RNA synthesis**
Echinocandins	**Caspofungin** **Micafungin** **Anidulafungin**	**Glucan synthesis**

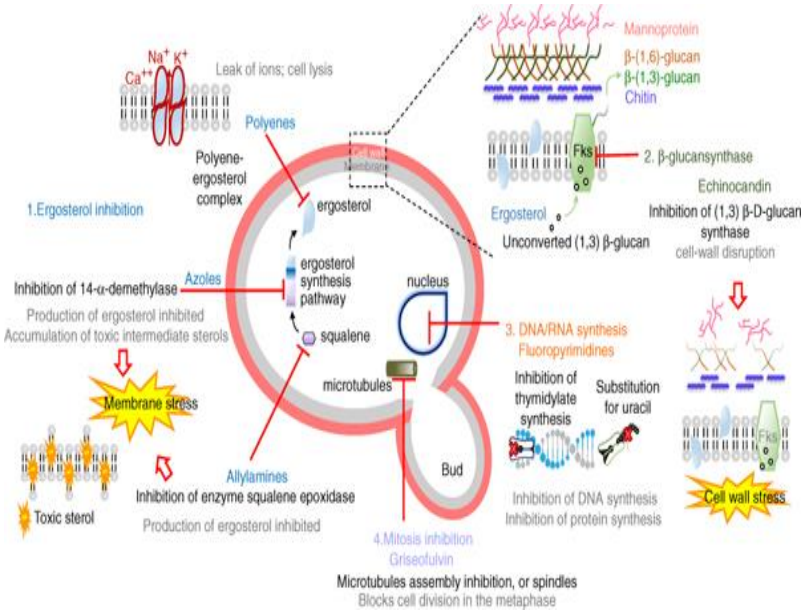


Fig 5 :Mechanism of action of antifungal drugs

Source: http://www.doctorfungus.org/thedrugs

Treatment of candidiasis depends on the site of the infection and the virulence of the organism [12].

- Mucocutaneous candidiasis – topical or systemic depending on the site
- Candidemia and deep seated infections – Systemic treatment [35].

The choice of the antifungal also depends on the host factors.

The treatment should be initiated only after the antifungal susceptibility testing. But for the patients in ICU the empirical therapy can be started in whom the presence of multifocal *Candida* colonization posses a high risk for invasive candidiasis and also in patients who has fever of unknown origin that is refractory to broad spectrum antimicrobials[12].

The selection of antifungal agents mainly depends on the species. As certain species are intrinsically resistant to certain antifungals [25].

Eg:

- *C.krusei* – intrinsically resistant to fluconazole and ketaconazole
- *C.lusitaniae* to amphotericin B

Standardization of in vitro susceptibility tests by the Clinical Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) are highly useful [72].

Table 11 : Methods used for antifungal susceptibility testing of *Candida*.

• Broth macro & micro dilution[73]
• Calorimetric microdilution[74]
• Sensititre yeast one test panel [75]
• Agar macrodilution
• Agar diffusion
  o Disk diffusion
  o E- test
  o Neo sensitabs[76]
• Flow cytometry[77].

Antifungal susceptibility has become important in clinical laboratory due to:

- Increase in the incidence of candidial infection in the last 15 yrs
- More frequent and prolonged use of antifungal agents.

- Increased emergence of resistance to the antifungals

3.9.Antifungal resistance [78]:

The emerging phenomenon of antifungal resistance is primarily a concern for invasive candidiasis. The knowledge about the antifungal resistance is less when compared to that of the antibiotic resistant bacterial infections, as they are the threat to public health. Thus, importance for understanding the emergence of antifungal resistance, awareness among medical and public health communities about these infections, and methods used to prevent and control them should be highlighted.

The changing epidemiology of *Candida* infection has been partly attributed to the selection of less sensitive *Candida* strains by the widespread use of the azole fluconazole as a prophylactic and therapeutic agent.

A study by Pfaller et al states that flucanozole is appearing resistant in *Candida albicans*[79].

Centre for Disease Control and Prevention (CDC) states that 7% of all *Candida* bloodstream isolates most of which are *Candida glabrata*. CDC's surveillance data indicate that the proportion of *Candida* isolates that are resistant to fluconazole has remained fairly constant over the past twenty years. In contrast, echinocandin resistance appears to be on the rise, with approximately 1% of all *Candida* isolates tested at CDC showing echinocandin resistance[80]. The antifungal resistance is divided into

- Clinical resistance
- Cellular or in- vivo resistance

3.9.1.Clinical resistance

It occurs in patients with severe immunosuppression like HIV. It is a result of low level of drugs in serum and or tissues caused by poor patient adherence, drug interaction that decreases antifungal levels.

3.9.2. Cellular or in –vivo resistance

It is independent of host. The pathogenic strains are less responsive to the standard dose of the drug.

It is divided into

- Primary or intrinsic
- Secondary or acquired

3.9.2a) Primary resistance:

It is demonstrated in organisms which are naturally resistant to antifungals. Eg : *C.krusei* intrinsically resistant to fluconazole

3.9.2b) Secondary resistance:

Initially the isolate is susceptible but later becomes resistant to an antifungal agent. Most commonly seen in HIV patients.

3.9.3. Mechanisms of resistance:

Resistance to azoles:

It is due to

- Changes in the sterol components of plasma membrane
- Genetic changes in ERG11gene encoding Lanosterol demethylase
- Alteration of enzymes involved in Ergosterol biosynthesis
- Drug efflux mechanism

Resistance to Amphotericin B:

It is poorly understood, probably due to changes in sterol components of plasma membrane and lipid composition of cell membrane.

Resistance to Flucytosine:

Deficiency of cytosine permease, cytosine deaminase, UMP pyrophosphorylase and loss of feedback regulation leading toincreased synthesis of pyrimidines.

Resistance to Echinocandins :

Due to mutations in FKS genes encoding 1,3 -D glucan synthase.

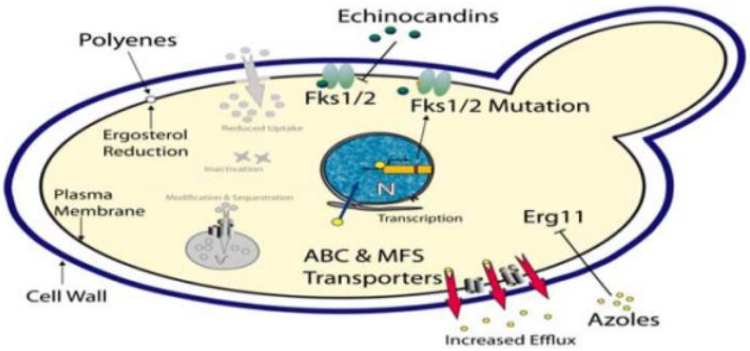


Fig 6: Mechanism of resistance in antifungal drugs

Source : http://www.mdpi.com/1424-8247/4/1/169/html

MATERIALS AND METHODS

4.0 MATERIALS AND METHODS

4.1.1. Design of study:

Cross sectional analytical study

4.1.2. Setting:

Department of Microbiology of a tertiary care hospital.

4.1.3. Period of study:

February 2015 –May 2016

4.1.4. Material:

All clinical samples like blood, urine, sputum, nail scrapings, high vaginal swab, tracheal aspirate and other body fluids received in the laboratory for culture.

4.1.5. Inclusion criteria:

All samples clinically suspected of candidiasis

4.1.6. Exclusion criteria:

Samples from patients with history of antifungal treatment during the last 6 months.

4.1.7. Data Collection:

All data were entered into Microsoft xcel spread sheet.

4.1.8. Statistical analysis:

Statistical analysis was carried out using SPSS software version 16.0. Variables are analysed for frequencies and percentages. Chi- square test was used as test of association, for which p value was calculated. Significance level was set as p value of 0.05. Fisher's Exact was used as appropriate.

4.2. Ethical committee approval:

Institutional Ethical committee approval was obtained before the start of the dissertation and the approval certificate is enclosed.

4.3. Collection of various clinical samples:

4.3.1. Respiratory tract samples:

Sputum sample was collected in a wide neck dry sterile container after proper instruction to the patient. Using the inoculation loop, purulent portion of the sputum was smeared on a clean, grease free, scratch free glass slide. The smear was then air dried, fixed and stained by the Gram technique. Samples were then microscopically examined for the Gram positive budding yeast cells and pseudohyphae.

The endotracheal tube tips were received in sterile containers. The tips including the bore were washed with ~0.5ml sterile peptone water. This was vortexed thoroughly and the resulting suspension was used to inoculate the plate for culture.

By instilling a small amount of sterile physiological saline into the bronchial tree and withdrawing the fluid, the broncho alveolar washings were obtained. A deeper sampling of desquamated host cells and secreations were also obtained via bronchoscopy by BAL.

For all these samples Grams smear was made and examined microscopically for the Gram positive budding yeast cells and pseudohyphae.

4.3.2. Pus & wound swabs:

Using a sterile technique, 5 ml of pus was aspirated with syringe and needle and transferred to a leak-proof sterile container.

For wound swab, the area was wiped with sterile normal saline or 70% alcohol and the swab was taken along the leading edge of the wound. Two swabs are

taken. One swab was used for culture and other for direct smear examination by Gram's stain.

Grams smear was made and examined microscopically for the Gram positive budding yeast cells and pseudohyphae.

4.3.3. Urine:

After washing the hands with soap and water, the female patients were instructed to clean the area around the urethral opening with soap and water, after cleaning, the area was dried by using a sterile gauze pad, and then the mid stream urine was collected with the labia held apart. In case of male patients, the same procedure was followed after retracting the foreskin.

Clean catch midstream urine was collected in a sterile, dry, leak proof, transparent screw capped container. The urine was processed within 2 hrs.

Catheter Collection:

The area was disinfected before proper collection of samples. Urine samples were aspirated using a sterile syringe and needle (gauge no.28), through the soft rubber connecter between catheter and collecting tubing.

The macroscopic appearance of the urine was noted. Then the urine is examined for the pus cells, budding yeast cells and pseudohyphae by wet mount preparation.

4.3.4. Blood:

Blood culture bottles containing Brain Heart Infusion Broth was examined daily for turbidity.

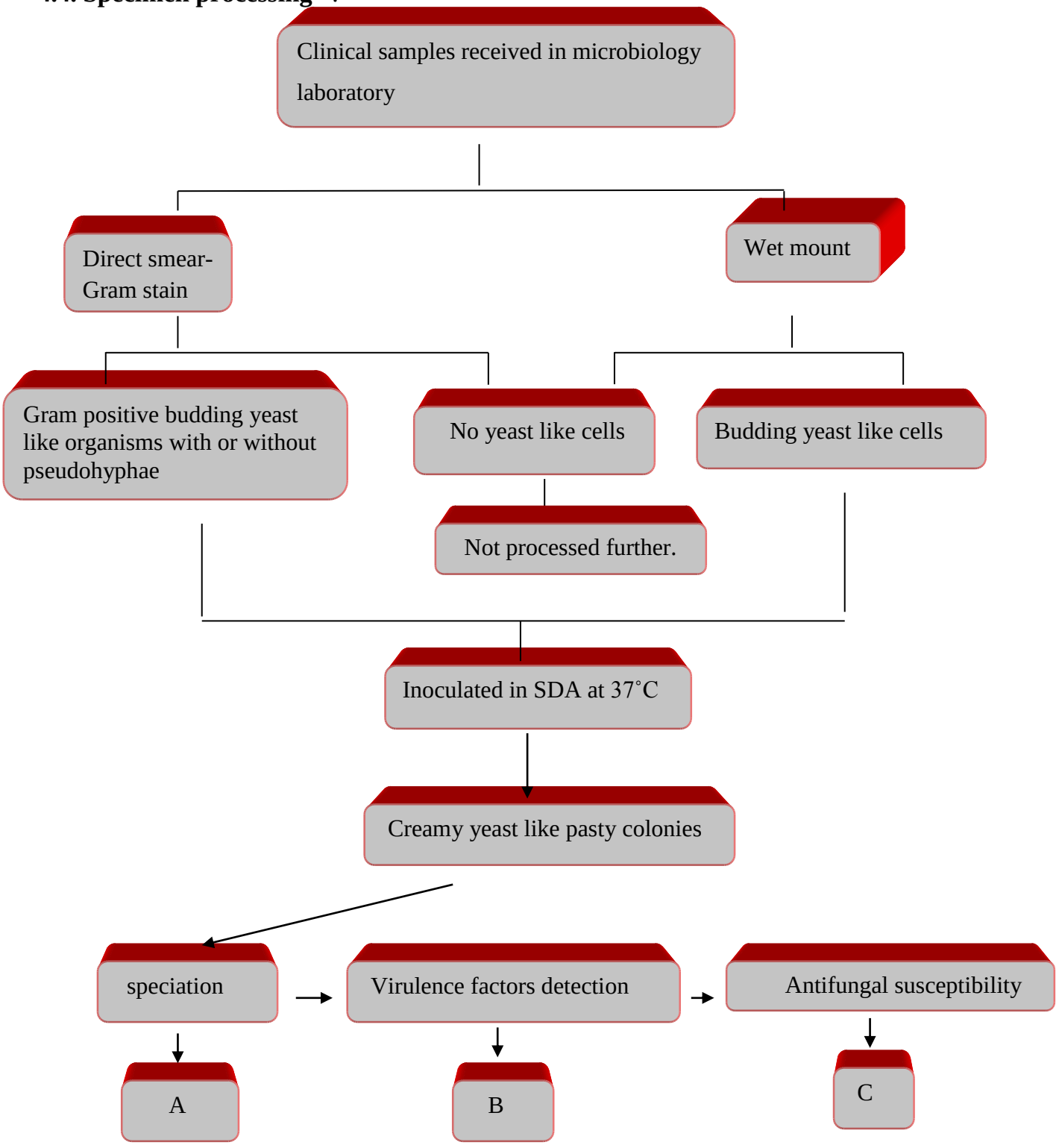
4.3.5. High vaginal swab:

Mucus was removed by gently rubbing the area with the cotton ball. The vaginal swab were made of cotton or rayon that has been treated with charcoal. The swab was inserted into the vaginal canal and rotated and moved from side to side for 30 seconds before removal. Two swabs were taken. One swab was used for culture and other for direct smear examination by Gram's stain.

4.3.6. Nail scrapings:

The affected nail was cleaned with 70% alcohol and allowed to dry for few seconds. Using no 15 surgical blade the nail was scraped in a sterile petri plate.

4.4. Specimen processing :



A)Species identification tests

5. Germ tube test.
6. Dalmau technique
7. Growth at 45^0C
8. CHROM agar
9. Sugar fermentation & assimilation.

B)Virulence factor detection

1. Biofilm formation – test tube method
2. Proteinase activity
3. Phospholipase activity - egg yolk medium
4. Coagulase activity

C)Antifungal susceptibility testing – disk diffusion methods

4.5.1 Wet mount:

Wet mount was done on urine and vaginal swab samples. Specimen was transfered on to the glass slide and cover slip was placed over it. The slide was examined first under the low power 10x and then high power 40x.Budding yeast cells with or without Pseudohyphae and yeast were looked for in the samples and then were processed.

4.5.2. Gram stain:

Gram stain was done using standard method. With the inoculation loop, clinical sample was transferred on to a clean glass slide and a thin smear was made. Then the smear was heat fixed by passing the slide over the flame. The smear was stained by Gram's Method and examined under 100X oil immersion field. Gram positive oval yeast like budding cells with pseudohyphae were visualized in relevant samples.

Then those samples were inoculated on SDA with chloramphenicol.

Clinical details about the patient were obtained and recorded. History, clinical findings, Co-morbid conditions, Surgical interventions and results of relevant investigations were also recorded. Ethical clearance was obtained.

4.6 Culture methods [3,12]:

The samples were processed on Sabourauds dextrose agar (SDA) with Chloramphenicol.

The clinical samples were inoculated in the SDA slopes and incubated at 37°C. The slopes were examined regularly from 2nd day onwards up to 3 weeks to give a negative report.

Colonies which appeared cream coloured, pasty and smooth were examined for Gram positive budding yeast cells by Gram's stain to confirm them as *Candida* species.

4.7 Species Identification method:

4.7.1 Germ tube test (Reynolds Braude Phenomenon):

A small portion of an isolated colony was suspended in a test tube containing 0.5 ml of rabbit or human plasma or serum. The test tube was incubated at 37°C for 2 hours. A drop of yeast suspension was placed on a microscope slide, overlaid with a coverslip and examined microscopically for the presence of germ tubes which are long tube like projection from yeast cells. Isolates producing germ tubes were presumptively identified as *C.albicans* or *C.dubliniensis* [81,82].

4.7.2. Growth at 45 °C:

Colonies inoculated in SDA were kept at 45°C in a waterbath. *C.albicans* were able to grow at this temperature while *C. dubliniensis* did not grow.

4.7.3. Cornmeal agar (CMA) [3]:

The media was prepared as per the manufacturer's instruction, autoclaved and poured in Petri dish. Heavy inoculums of yeast were streaked across the plate and cover slip was placed over it. The streak line should project beyond the cover slip. The cover slip was placed to create partial anaerobic environment. Plates were incubated for 48 hours at 25°C. Colonies were observed at the interface of the cover slip and line of inoculum under low power and high power subsequently for chlamydospores, blastospores, pseudohyphae and hyphae.

Table 12. Microscopic identification of *Candida* species by Dalmau technique	
C.albicans	Pseudomycelium is produced. Large clusters of blastoconidia are formed in grape like clusters, along the length of hyphae. Sessile, intercalary and many terminal chlamydospores are seen [3].
C.tropicalis	Abundant pseudohyphae with lots of branching and blastoconidia either singly or in clusters are seen [3].
C.krusei	Long, slender, elongated tree like branching pattern are seen. Branching occurs from the junction between cells resembling "crossed match sticks[3].
C.glabratta	Absence of pseudohyphae Yeast cells measuring 2-4μm forms a distinctive feature of this species [3].
C.guilliermondii	Budding cells spherical to broadly ellipsoidal 3-6x2-4 μm. True hyphae are absent and pseudomycelium may be present. Blastoconidia are seen in small chains or in clusters and ovoid in shape [3].
C.parapsilosis	Pseudohyphae are long thin curved with clusters of blastospores along them. Occasional giant cells are seen[3].

C.kefyr	Pseudohyphae are fragile with long cells. They fall apart and form palisades. Few blastoconidia may be seen [3].

The findings were recorded and tabulated**.**

4.7.4. CHROM agar [12]:

It is a selective media for the isolation and identification of different species of *Candida*. Media was prepared as per the manufacturer's instruction and was dispensed in petri dishes after being allowed to cool slightly. Isolates were plated directly from SDA to HI-Chrom agar and incubated at 30°C for 48 hours. The various species of *Candida* were identified by their colony color, size, texture, and presence of color diffusion into the surrounding agar presumptively in 48hrs

C. albicans ATCC 90028 was used as the control strain.

Table13: Colours produced by various *Candida* species

***Candida* species**	**Colours**
C.albicans	Green
C.dubliniensis	dark green
C.tropicalis	dark blue
C.krusei	Dry pink
C.parapsilosis	white to pale pink
C.glabrata	white to pink

The findings were recorded and tabulated**.**

4.7.5. Sugar fermentation and sugar assimilation:

i) Sugar fermentation [12]:

Liquid medium containing peptone, 2% sugar and indicator was poured in a test tube (~5ml) and Durham's tube was placed into each tube. Heavy inoculums of yeast colonies were suspended into each tube and incubated at 25°C for 1 week. The

tubes were examined at 48- 72 hours interval for acid (pink color) and gas (in durham's) production. *C.albicans* ATCC 90028 was used as the control strain. Species identification was done by referring the table given below

Table 14 :Fermentation of different sugars by different species

Sugars	***C.albicans***	***C.tropicalis***	***C.krusei***	***C.parapsilosis***	***C.glabrata***	***C.guilllermondii***	***C.lusitaniae***	***C.kefyr***
Glucose	AG	AG	AG	AG	AG	AG	AG	AG
Lactose	-	-	-	-	-	-	-	AG
Sucrose	AG	-	-	-	-	AG	AG	AG
Maltose	AG	AG	-	-	-	-	-	-
Galactose	AG	AG	-	-	-	AG	AG	AG
Trehalose	AG	AG	-	AG	AG	AG	AG	AG

The findings were recorded and tabulated**.**

ii) Sugar assimilation (Auxonographic techniques) [65]:

18ml quantities of agar and distilled water was dispensed in screw capped tubes. Autoclaved at 121°C for 20 minutes and stored at 4°c. A heavy inoculums yeast suspension was prepared from 24 hours old culture in 2ml Yeast Nitrogen Broth. The prepared suspension was poured into 18 ml of molten agar cooled at 45°C, and then poured into a 90mm Petri plate. The Petri plate was set at room temperature until the agar surface hardens. Sugar discs were placed in a circle with sterile forceps, such that they were at least 30mm was present between centers of each disc and was incubated at 37°C 3-4 days. Presence of growth around each disc

indicates assimilation of respective sugars. *C.albicans* ATCC 90028 was used as the control strain. Species identification was done by referring the table given below.

Table 15. Sugar assimilation by different *Candida* species

Species	Glucose	Lactose	Sucrose	Maltose	Galactose	Melibiose	Cellibiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol
C.albicans	+	-	+	+	+	-	-	-	+	-	+	-
C.tropicalis	+	-	+	+	+	-	+	-	+	-	+	-
C.krusei	+	-	-	-	-	-	-	-	-	-	-	-
C.parapsilosis	+	-	+	+	+	-	-	-	+	-	+	-
C.glabrata	+	-	-	+	-	-	-	-	-	-	+	-
C.guilliermondii	+	-	+	+	+	+	+	-	+	+	+	+
C.lusitaniae	+	-	+	+	+	-	+	-	+	-	+	-
C.kefyr	+	+	+	-	+	-	+	-	+	+	-	-

The findings were recorded and tabulated**.**

4.8. Virulence factor detection:

4.8.1.Biofilm formation [83]:

Biofilm formation was detected in all the isolates by using a method proposed by Branchini *et al*. A loopful of 24-48 hours old organism were taken from the SDA plate and inoculated into a tube containing 10 ml sabouraud's liquid medium added with glucose -final concentration of 8%. The tubes were incubated at 37°C for 48 h after which the broth is aspirated out and the walls of the tubes were stained with 1% safranin solution. Biofilm formation was graded as:

- Negative – 0
- Weak positive – 1+

- Moderate positive – 2+
- Strong positive – 3+

4.8.2. Proteinase factor detection [84]:

The yeast suspension was made by inoculating few colonies on yeast extract peptone dextrose (YEPD) broth and incubated over night.

Bovine serum albumin agar base was prepared and autoclaved at 121°Cfor 20 minutes. After cooling to 50°C it was mixed with 1% bovine serum albumin. Then medium was poured in sterile 90 mm Petri dish. Sterile filter paper disc were placed on the four outer quadrant of Petri dish and soaked with YEPD broth containing overnight growth. In the centre of Petri dish a disc with uninnoculated YEPD broth was placed which served as a control.

The plates were incubated at 37°C for 48 hours. Then the plates were stained with 1% amidoblack solution for 5 minutes and washed with tap water.

Zone of clearing surrounding the disc was noted against ordinary light.

Proteinase activity was graded as follows

Grade I – Visible proteolysis limited 1-2 mm

Grade II - Visible proteolysis more than 2mm

4.8.3. Phospholipase activity [85,86]:

Phospholipase activity was estimated on Egg yolk agar by grading the zone of precipitation by slightly modified method of Samarnayke et al.

Egg yolk medium was prepared as per manufacterer's instruction and was Autoclaved at 121°Cfor 20 minutes and after cooling up to 50°C ,10% egg yolk emulsion was added. Extracellular phospholipase activity was detected by inoculating

10µl of yeast suspension (~108 cfu/ ml) on a sterile paper disc placed over the four quadrants of the medium. The plate was incubated at 37°C for 48 hours.

Phospholipase activity (Pz) was calculated by:

$$Pz = \frac{\text{colony diameter}}{\text{Colony diameter + zone of precipitation}}$$

Based on the above formaula, phospholipase activity is classified into 5 types

- Pz value = 1 means - negative
- Pz value < 0.90-0.99 = weak phospholipase activity (+)
- 0.80-0.89 = poor phospholipase activity (++),
- 0.70-0.79 = moderate phospholipase activity (+++)
- <0.70 = intense phospholipase activity (++++)

4.8.5. Coagulase activity [87]:

Coagulation production was detected by the method of Yigit et al. Approximately 0.1ml of overnight culture was aseptically inoculated into a tube containing 500µL of rabbit plasma. The tubes were incubated at 35°C and observed for clot formation after 2,4, 6 and 24h.

The presence of clot that was not be resuspended by gentle shaking indicated positive coagulase test.

4.9. Antifungal susceptibility testing :

Antifungal Susceptibility Testing for *Candida* isolates was done by

- Disc diffusion method, as per CLSI Guidelines on Antifungal Susceptibility testing in M-51A document.

Disc diffusion method:

Glucose methylene blue (GMB) stock solution was prepared

GMB stock solution was prepared and Autoclaved for 15 minutes at 121 °C. The solution was stored at room temperature and were not refrigerated as this may cause precipitation.

Mueller-Hinton agar with 2% glucose and 0.5 µg/ml methylene blue

Mueller-Hinton agar was prepared according to the manufacturer's instructions.

GMB solution with methylene blue was poured into the freshly prepared and cooled Mueller Hinton agar medium and then they were poured into plastic, flat-bottomed petri dishes to a depth of approximately 4 mm. Then agar medium was allowed to cool to room temperature and stored at refrigerator temperature (2 to 8 °C)

The agar medium should have a pH between 7.2 and 7.4 at room temperature.

Inoculum preparation:

Inoculum was prepared by picking five distinct colonies from a 24-hour-old culture of *Candida* species and the colonies were suspended in 5 ml of sterile normal saline. The turbidity was adjusted equivalent to 0.5 McFarland standards (1x 10^6 to 5 x 10^6 cells/ml) resulting in semi-confluent growth.

Inoculation of the agar plate:

A sterile cotton swab was dipped into the suspension and rotated several times. The excess fluid was removed from the swab by pressing firmly against the inside wall above the fluid level. Then a lawn culture was made on the dried surface

of the agar by streaking the cotton swab according to the standard three directional method.

The plate was left open for 3 – 5 minutes, allowing excess moisture to be absorbed, and then the antifungal disc were dispensed onto the plate.

Application of disks to inoculated plates:

Antimicrobial discs were dispensed onto the surface of an inoculated agar plate by means of a sterile forceps and pressed down. The discs were evenly distributed on the plate with a distance of 2.5 cm from centre to centre of the discs.

Incubation:

Plates were inverted and incubated at 35°C ±2°C within 15 minutes after placing the discs. The zone is read after 20-24 hours of incubation. If no visible growth with particular strains, the plate is re-incubated for 48 hours and then read. Zone of inhibition was measured at the point where there was prominent reduction in growth.

Table 16 :Zone size interpretive chart for *Candida* antifungal susceptibility testing[88]

DRUGS	S	SDD / I	R	Source document
Fluconazole	≥19	15-18	≤14	CLSI M44-A2
Voriconazole	≥17	14-16	≤13	
Ketoconazole	≥28	27-21	≤ 20	Arbitrary
Itraconazole	≥15	10-14	≤ 9	
Amphotericin-B	≥19	15-18	≤14	

The findings were recorded and tabulated**.**

Table 17. Recommended QC for different antifungals				
Antifungal agent	**Disc strength (µg)**	**Expected zone diameter (mm)**		**Source document**
		***Candida albicans* ATCC 90028**	***Candida parapsilosis* ATCC 22019**	
Fluconazole	25	28-39	22 – 33	**CLSI M44-A2**
Voriconazole	1	31-42	28 – 37	
Amphotericin-B	20	10-15	10-17	**HIMEDIA manual**
Itraconazole	10	16-20	11-18	
Ketoconazole	10	20-32	14-29	

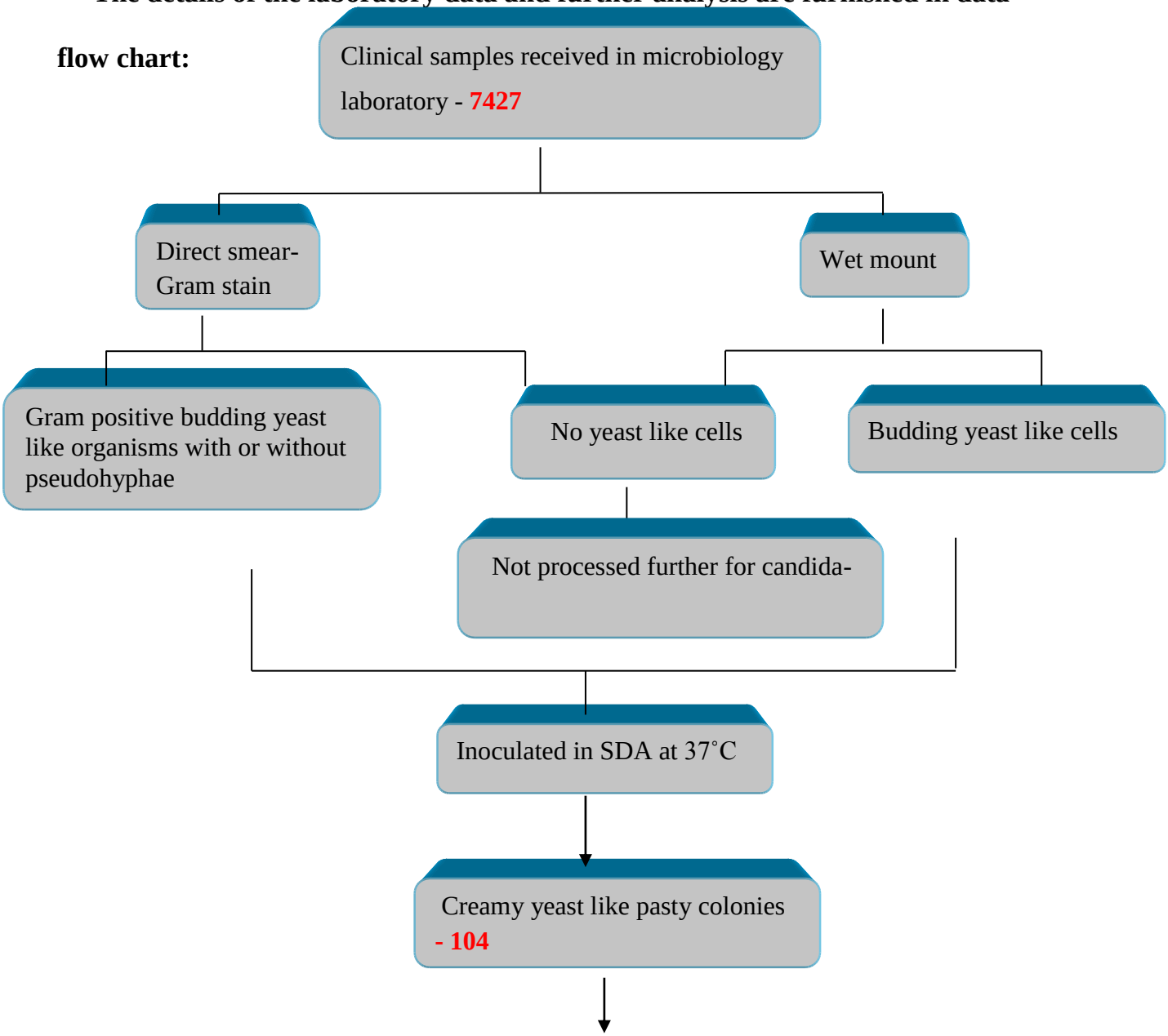
Quality control stains were used for different antifungal drugs**.** The findings were recorded and tabulated**.**

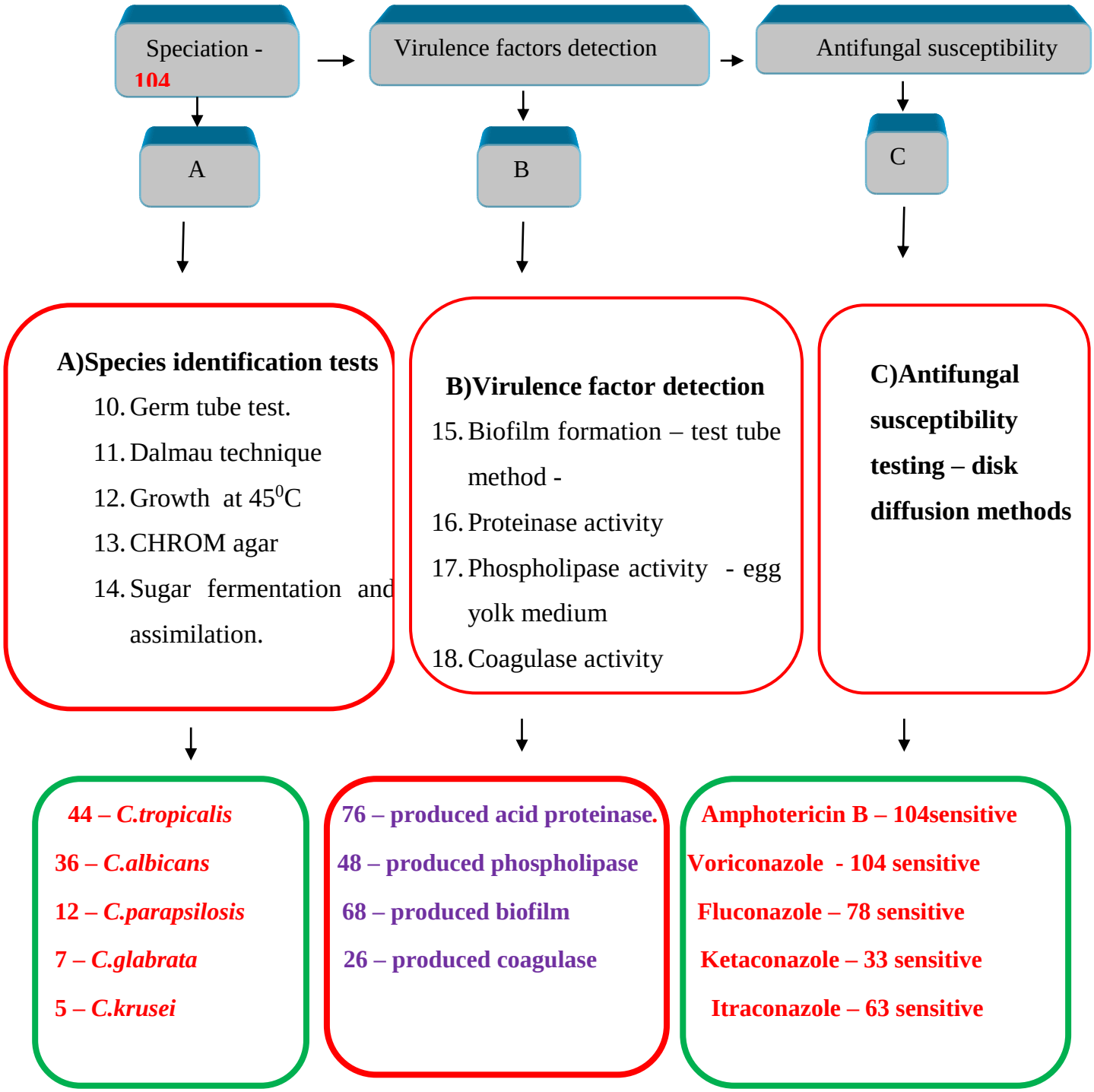
RESULTS

5.0 RESULTS

The study entitled "Speciation, Virulence Factors detection and Antifungal Susceptibility testing of *Candida isolated* from heterogenous clinical samples" was carried out in the department of Microbiology, Chennai medical College Hospital and Research centre. The observations made are presented in the ensuing pages with regard to microbiological and its clinical association.

The details of the laboratory data and further analysis are furnished in data flow chart:





A total of 7,427samples were received in Microbiology Laboratory during the period of February 2015 to May 2016 for culture and sensitivity and their distribution in relation to the source is depicted in Figure 7

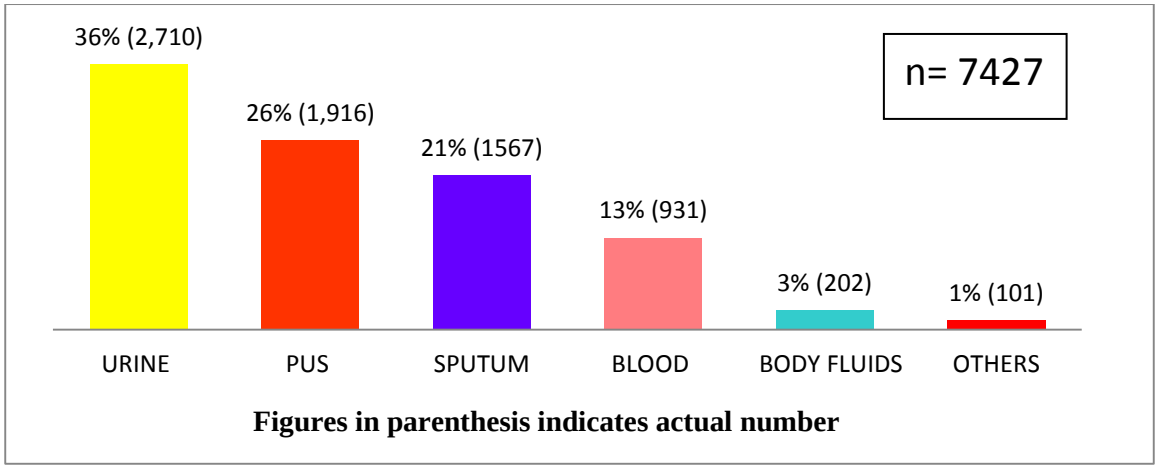


Fig 7: Samples received in Microbiology Laboratory

The isolation of the organisms in relation to clinical sample revealed that *Candida* isolation were rare from blood and body fluids, but little more from other samples such as pus, sputum and urine.

Table : 18. Analysis of isolates

Clinical sample	**Sample received**	**No growth**	**Bacterial growth**	***Candida***	**Ratio between bacteria and *candida***
Urine	2710	1059	1608	43	38:1
Pus & others including swabs	1916	487	1408	21	67:1
Sputum	1567	101	1437	29	49:1
Blood	931	602	328	1	328:1
Body fluids	202	102	96	4	24:1
Others	101	35	62	4	16:1
Total	7427	2386	4937	104	

Of 7427 samples received in the microbiology laboratory, 5041(68%) yielded positive growth. Out of these 5041, *Candida* constituted 104 (2.05%) and others were bacterial isolates. The details are given in Figure 8.

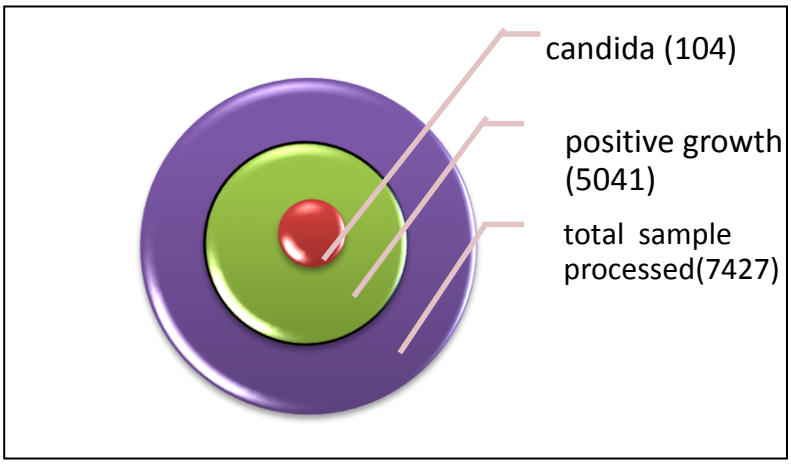


Fig 8 : Distribution of *Candida* in a tertiary care hospital

These 104 *Candida* isolates were taken for detailed work up.

5.1. Age Distribution:

In our study, the mean age was 51.75 with a standard deviation of 18.172 years. The youngest patient being two and the oldest was 85years. The distribution of the isolates in relation to the age group is provided in the Figure 9.

During the study period no isolates were from the age group 6 and 19 yrs. Isolation above 60 years was more and significant statistically ($p < 0.01$)

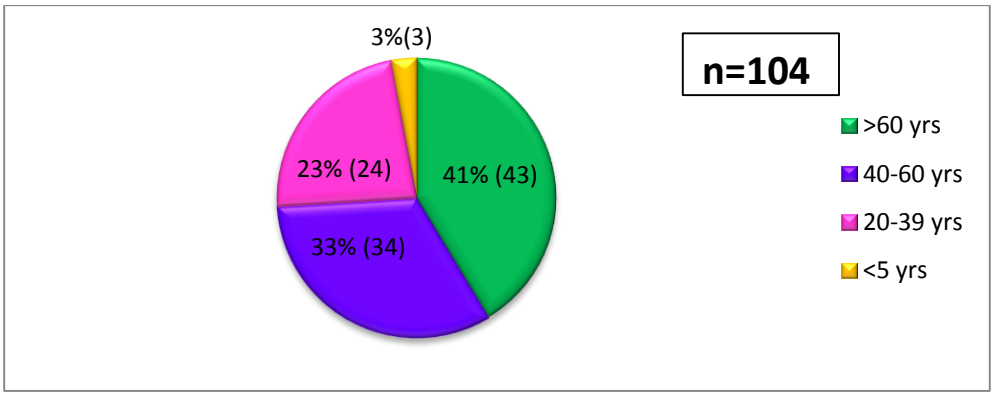


Fig 9: Age distribution

5.2. Gender wise Distribution:

Out of 104 samples, 53(51%) were from males and 51(49%) from females. Genderwise distribution is given in Figure 10. There was no statistical difference with regard to gender.

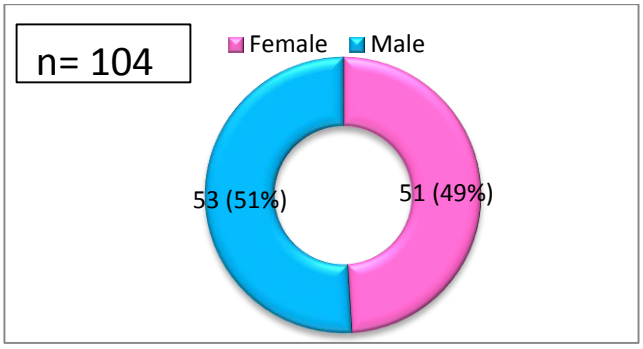


Fig 10:Male female ratio

5.3.Sample wise distribution of *Candida*:

Out of 104 *Candida* isolates 34.6 % were from Catheterized urine sample followed by sputum (27.8%) and vaginal swab (8.6%). The *Candida* isolates in relation to different systems are urogenital, respiratory, gastrointestinal and others were 52, 33,9,10 respectively. Their distribution in relation to clinical samples are provided in Figure 11

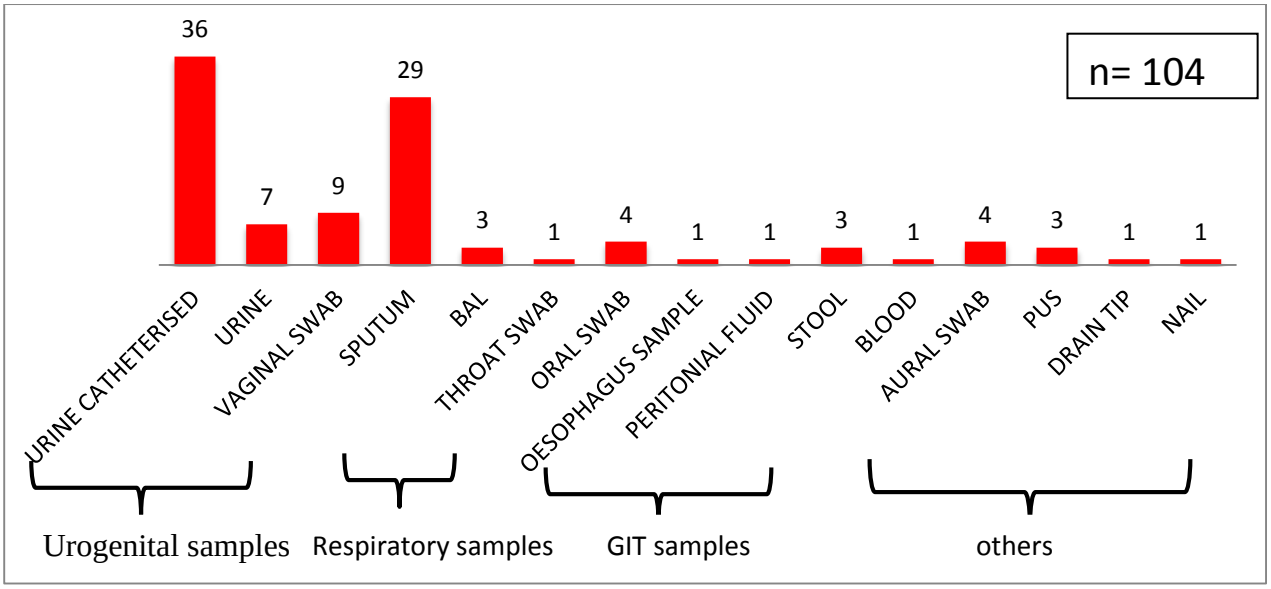


Fig 11: Isolate distribution among heterogenous clinical samples

5.4.Ward wise distribution of *Candida* isolates:

The ward wise distribution of *Candida* isolates is given in Figure 12. It was observed that out of 104 *Candida* isolates, 92 of *Candida* were isolated from medical wards and the remaining from the surgical wards. The difference is statistically significant (p <0.001).

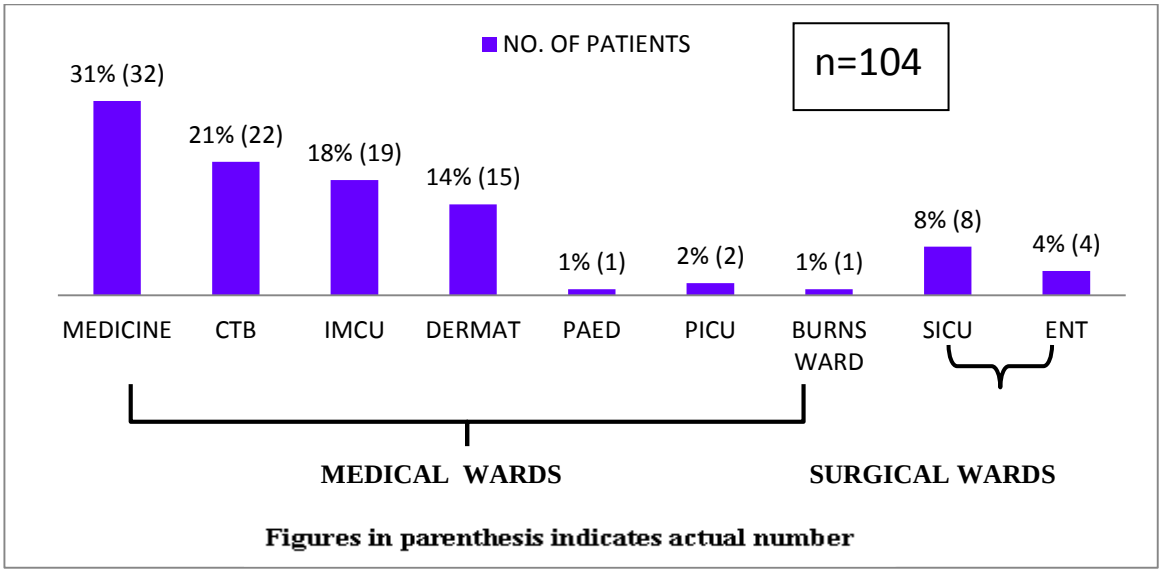


Fig 12: Graph indicating ward wise distribution of *Candida* isolates

5.5. Inpatient and outpatient distribution of *Candida*:

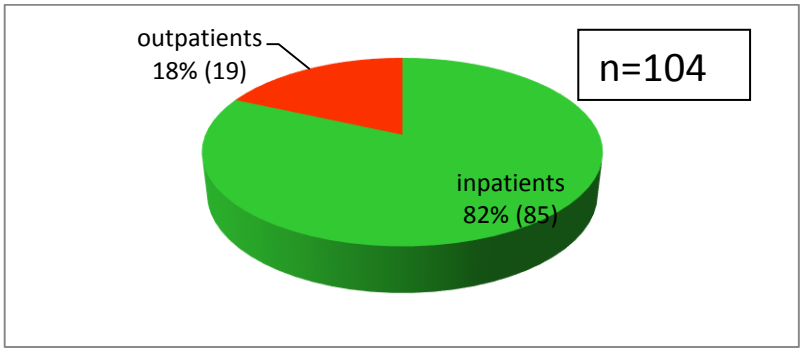


Fig 13: Distribution of *Candida* among inpatients and outpatients

The above graph depicts the *Candida* isolates were more from in patients than outpatients. This difference is significant statistically ($p<0.001$).

5.6.Association between Isolates and co morbid conditions:

The Figure 14 shows the association between the isolates and the co-morbid conditions.Of these 104 *Candida* isolates, 71 (68%) were from patients suffering from diabetes mellitus and 14 (13%) from those who had pulmonary tuberculosis.

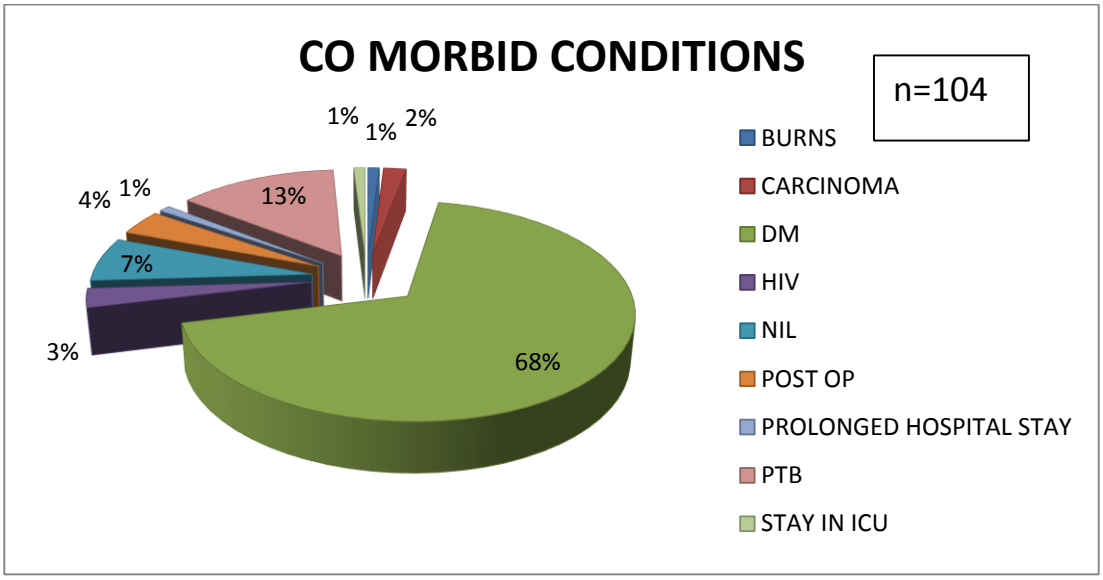


Fig 14:Graph indicating the co-morbid conditions

5.7. Species identification:

5.7.1. Identification of *Candida* species based on Germ Tube Test:

These 104 *Candida* isolates when subjected to Germ Tube Test (GTT), GTT was positive in 36 (35%) and negative in the remaining 68 (65%). The results of GTT positive and negative is provided in the Figure 15.

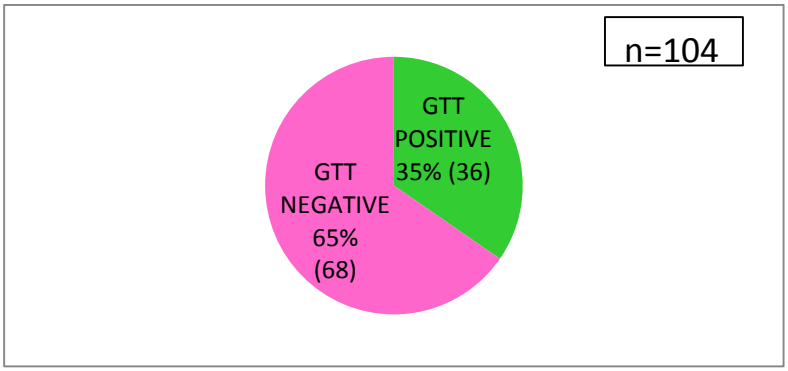


Fig15: Speciation by Germ Tube Test

5.7.2.Species identification by growth at temperature 37^0C & 42^OC:

All the 104 *Candida* isolates grew at 37^0C, only 36 of them grew at 42^0C also.

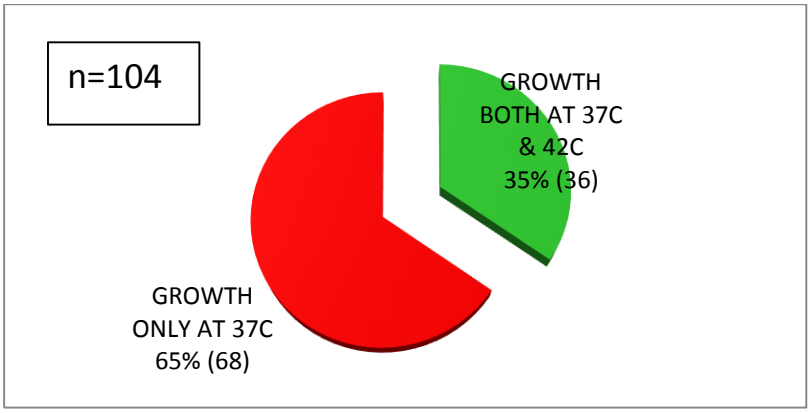


Fig 16: Speciation by growth at temperature 37^0C & 42^OC

5.7.3. Distribution of *Candida* albicans and *Candida* non albicans species:

In this study, *Candida* albicans was identified by the GTT and growth at both 37^0C and 42^0C which constituted 36 (35%) and the remaining 68 (65%) were *Candida*

non albicans. Thus a large number of isolates belonged to *Candida* non albicans, and the distribution is furnished in Figure 17

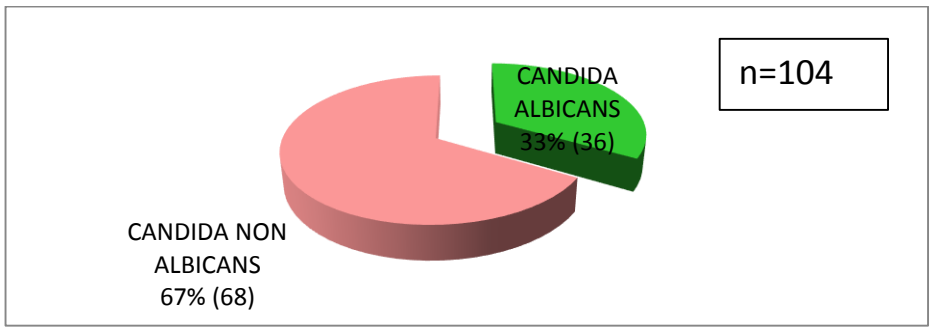


Fig :17 Distribution of *Candida* albicans and *Candida* non albicans

5.7.4.Species identification by Dalmau technique:

Using Dalmau technique for these 104 *Candida* isolates, 44 were identified as *C.tropicalis*, 36 as *C.albicans*, 12 as *C.parapsilosis*,7 as *C.glabrata* and 5 as *C.krusei*. The observations made while using Dalmau technique are described in Table 19.

Table 19: Speciation by Dalmau technique

Observation	Species	No (%)
Branching Pseudohyphae with blastoconidia singly or in clusters.	***C.tropicalis***	**44 (42.3)**
Pseudohyphae present.Clusters of blastoconidia. Terminal chlamydospores were present	***C.albicans***	**36 (34.6)**
Thin Pseudohyphae present. Giant cells were present.	***C.parapsilosis***	**12 (11.5)**
No pseudohyphae only yeast cells were present	***C.glabrata***	**7 (6.7)**
Branching were present from the junction between cells -crossed match sticks	***C.krusei***	**5 (4.8)**
Total		**104**

5.7.5.Species identification by CHROM agar:

All the 104 *Candida* isolates were checked for growth on CHROM agar and the colour changes were noted. The speciation was made according to the colour noticed and speciation is given in Table 20. Of these 104, two showed dark green colour and hence these 2 could not be considered as *Candida* albicans. However, these two when tested by other methods were found to be *Candida* albicans.

Table 20: Speciation by CHROM agar

COLOUR -CHROM AGAR	SPECIES	NO.(%)
Blue purple	***C.tropicalis***	**44 (42.3)**
Light green	***C.albicans***	**34 (32.6)***
Mauve	***C.parapsilosis***	**12 (11.5)**
Purple	***C.glabrata***	**7 (6.7)**
Pink	***C.krusei***	**5 (4.8)**
TOTAL		**102***

*- 2 Species of *Candida* gave dark green colour

5.7.6. Identification of *Candida* species based on sugar fermentation :

All the 104 *Candida* isolates were subjected to sugar fermentation and based on the fermentation of sugars (acid and gas production) the isolates were speciated and tabulated. The results are given in Table 21.

Since the fermentation of sugars vary with the species, further statistical analysis was not attempted.

Table 21: Species identification by sugar fermentation							
Species	**Number**	**Fermentation of sugars**					
		GLU	**LAC**	**SUC**	**MAL**	**GAL**	**TRE**
C.tropicalis	**44**	**44**	**-**	**44**	**44**	**44**	**44**
C.albicans	**36**	**36**	**-**	**-**	**36**	**36**	**36**
C.parapsilosis	**12**	**12**	**-**	**-**	**-**	**-**	**-**
C.glabrata	**7**	**7**	**-**	**-**	**-**	**-**	**7**
C.krusei	**5**	**5**	**-**	**-**	**-**	**-**	**-**
Total	**104**						

5.7.7.Identification of *Candida* species based on sugar assimilation:

All the 104 *Candida* isolates were subjected to sugar assimilation tests using 12 sugars. The assimilation of these individual sugars in relation to the species are furnished in Table 22.

In view of assimilation of sugars varying with the species, further statistical analysis was not attempted.

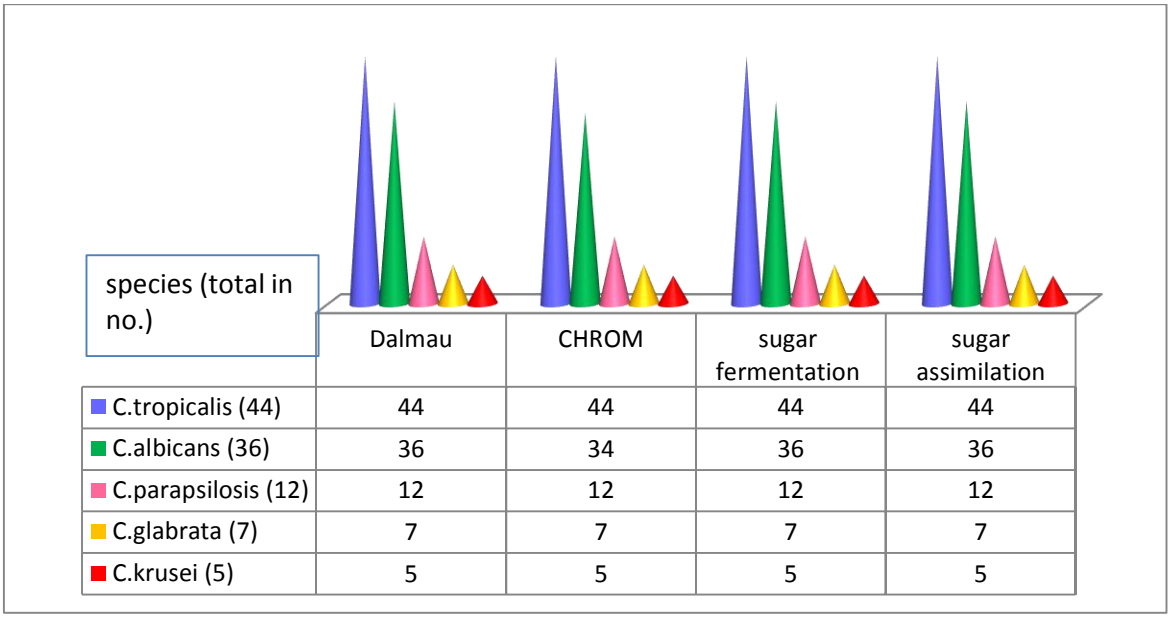
Table 22: Species identification by sugar assimilation													
Species	**Assimilation of sugars**												
	Total	**GLU**	**MAL**	**SUC**	**LAC**	**GAL**	**MEL**	**CEL**	**INO**	**XYL**	**RAF**	**TRE**	**DUL**
C.tropicalis	**44**	**44**	**44**	**44**	**-**	**44**	**-**	**44**	**-**	**44**	**-**	**44**	**-**
C.albicans	**36**	**36**	**36**	**36**	**-**	**36**	**-**	**-**	**-**	**36**	**-**	**36**	**-**
C.parapsilosis	**12**	**12**	**12**	**12**	**-**	**12**	**-**	**-**	**-**	**12**	**-**	**12**	**-**
C.glabrata	**7**	**7**	**7**	**7**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**7**	**-**
C.krusei	**5**	**5**	**5**	**5**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Total	**104**												

5.7.8. *Candida* distribution of species in relation to the tests:

Out of 104 *Candida* isolates, 44 were identified as *C.tropicalis*, 36 as *C.albicans*, 12 as *C.parapsilosis*, 7 as *C.glabrata* and 5 were identified as *C.krusei* by Dalmau technique, CHROM agar, sugar fermentation and sugar assimilation. The details are provided in Figure 18.

By CHROM agar we were able to ascertain colour for only 34 of the 36 *Candida* albicans. Two of other *Candida* albicans were confirmed by other speciation tests including GTT and growth at both 37^0C and 42^0C, showed dark green colour when subjected to CHROM agar.

Thus, Dalmau, sugar fermentation and sugar assimilation shows 100% sensitivity and hence was considered as gold standard, whereas the sensitivity of CHROM is 97.1% (compared with Dalmau)



species (total in no.)	Dalmau	CHROM	sugar fermentation	sugar assimilation
C.tropicalis (44)	44	44	44	44
C.albicans (36)	36	34	36	36
C.parapsilosis (12)	12	12	12	12
C.glabrata (7)	7	7	7	7
C.krusei (5)	5	5	5	5

Fig 18: Speciation by Dalmau, CHROM agar, sugar fermentation and sugar Assimilation

5.7.9.Species distribution:

The Figure 19 depicts the distribution of 104 *Candida* species isolated during the study. Among 104 *Candida* isolates, 44 were identified as *C.tropicalis*, 36 as *C.albicans*, 12 as *C.parapsilosis*,7 as *C.glabrata* and 5 as *C.krusei*.

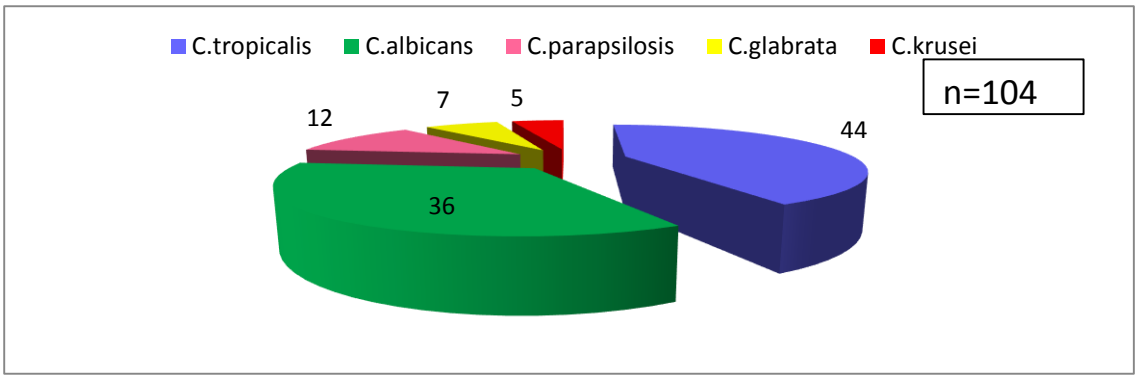
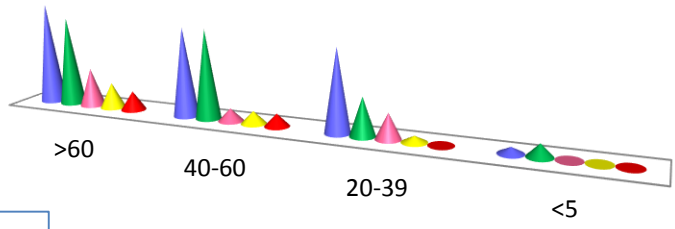


Fig 19:Species isolated

5.7.10.Agewise distribution of *Candida* species:

The distribution of *Candida* species with regard to age group is furnished in Figure 20. Out of 104 *Candida* isolates, C. glabrata was more common in age group >60yrs (57.10%). *C.krusei* was common among 40 – 60 yrs of age (40%) followed by *C.albicans* (38.9%). C. parapsilosis was common among 20 – 40yrs of age (33.3%). C. albicans was common among children <5 yrs (5.6%).



species (total in no.)	>60	40-60	20-39	<5
C.tropicalis(44)	16	14	13	1
C.albicans (36)	14	14	6	2
C.parapsilosis (12)	6	2	4	0
C.glabrata (7)	4	2	1	0
C.krusei (5)	3	2	0	0

Fig 20: Distribution of *Candida* species among different age groups

5.7.11.Sample wise distribution of *Candida* species:

Distribution of *Candida* species with regard to clinical samples is given in Table 23. Out of 104 *Candida* isolates , *C.tropicalis* were isolated predominantly from respiratory specimens (29.5%) followed by catheterized urine (27%) and vaginal swab (13.6%), *C.albicans* from respiratory specimen (38.8%), urine (38.8%), one blood and vaginal swab (5%), *C.glabrata* from urine (4 out of 7 isolates) and sputum (3out of 7) isolates, *C.parapsilosis* from urine (50%) and respiratory samples (25%) and *C.krusei* (all the five) only from catheterized urine samples.

Table 23: Distribution of *Candida* among heterogenous clinical samples																
	Genito urinary samples			Respiratory samples			Gastro intestinal samples				Others					
Name of the species of *Candida*	URINE-CATHETERISED	URINE	VAGINAL SWAB	SPUTUM	BAL	THROAT SWAB	ORAL SWAB	ESOPHAGOSCOPY SAMPLE	PERITONEAL fIFLUID	STOOL	AURAL SWAB	BLOOD	DRAIN TIP	NAIL	PUS	TOTAL ISOLATES
C.tropicalis	**12**	**2**	**6**	**10**	**2**	**1**	**4**	**0**	**0**	**2**	**3**	**0**	**1**	**0**	**1**	**44**
C.albicans	**10**	**4**	**2**	**13**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	**1**	**0**	**1**	**2**	**36**
C.parapsilosis	**5**	**1**	**1**	**3**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**12**
C.glabrata	**4**	**0**	**0**	**3**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**7**
C.krusei	**5**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**5**
Total	**36**	**7**	**9**	**29**	**3**	**1**	**4**	**1**	**1**	**3**	**4**	**1**	**1**	**1**	**3**	**104**

5.8.Virulence Factors:

Virulence of *Candida* sp was assessed by proteinase activity, phosphospholipase production, biofilm formation and coagulase production.

5.8.1Proteinase activity:

5.8.1.1.Proteinase activity among *Candida* species:

Out of 104 *Candida* isolates, 76 (73%) produced acid proteinase. Proteinase production was detected in 33out of 44 *C.tropicalis*, 30 out of 36 *C.albicans*, 6 out of 12 *C.parapsilosis*, 3 out of 7 *C.glabrata* and 4 out of 5 *C.krusei*. The details of proteinase production with regard to species is given in Figure 21.

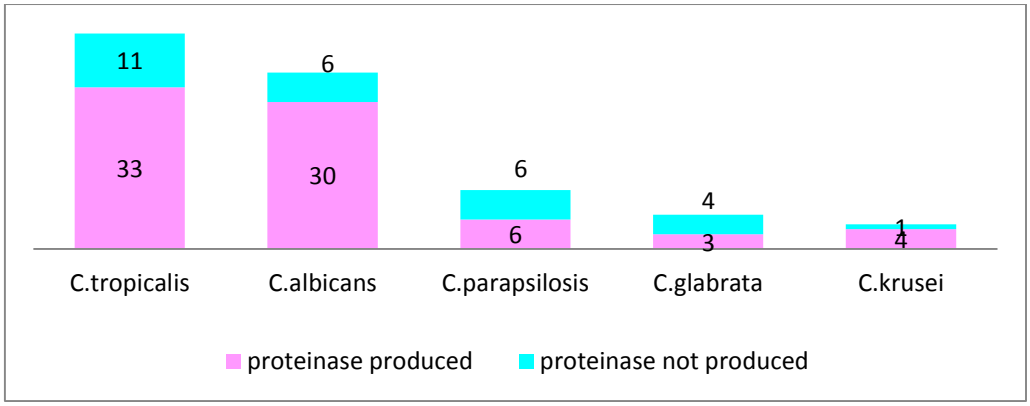


Fig 21 :Proteinase activity among various *Candida* species

5.8.1.2. Association of proteinase activity with the clinical samples:

The association of proteinase activity with regard to isolates belonging to various clinical samples is given in Table 24. In the present study, 30 out of 36 isolates of *Candida* from catheterized urine, 13 out of 29 sputum, 9 out of 9 vaginal isolates and single blood isolate produced proteinase. This difference is statistically significant with p value of < 0.0001.

Table 24: Association of proteinase activity with the clinical sample

Samples	Proteinase activity		
	Produced	Not produced	Total
Urine Catheterised	30	6	36
Urine	3	4	7
Vaginal Swab	9	0	9
Sputum	13	16	29
Bal	3	0	3
Throat Swab	1	0	1
Oral Swab	4	0	4
Oesophagoscopy Sample	1	0	1
Peritoneal Fluid	1	0	1
Stool	3	0	3
Aural Swab	2	2	4

Blood	**1**	**0**	**1**
Drain Tip	**1**	**0**	**1**
Nail	**1**	**0**	**1**
Pus	**3**	**0**	**3**
Total	**76**	**28**	**104**

5.8.2.Phospholipase production:

5.8.2.1.Phospholipase production among *Candida* species:

Out of 104 *Candida* isolates, 48 (46%) produces Phospholipase. Phospholipase production was detected in 14 out of 44 *C.tropicalis* (31.8%),32 out of 36 *C.albicans* (89.9%), 1 out of 12 *C.parapsilosis* ,1 out of 7 *C.glabrata* and this difference is statistically significant with p value of <0.0001.

The details of phospholipase production with regard to species is given in Figure 22.

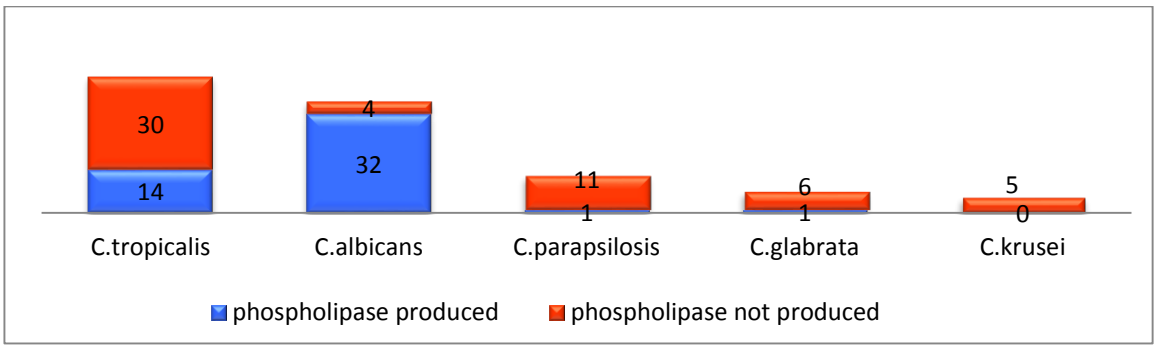


Fig 22 :Phospholipase production among various *Candida* species

5.8.2.2. Association of phospholipase activity with the clinical samples:

The association of phospholipase activity with regard to isolates belonging to various clinical samples is given in Table 25. In the present study, the phospholipase production was observed in 16 out of 36 catheterised urine sample, 2 out of 3isolates from pus, followed by 5 out of 9 vaginal swabs and this difference is statistically significant with p value of <0.0001.

Table 25: Association of phospholipase activity with the clinical sample			
Samples	Phospholipase production		
	Produced	Not produced	Total
Urine Catheterised	16	20	36
Urine	4	3	7
Vaginal Swab	5	4	9
Sputum	12	17	29
Bal	1	2	3
Throat Swab	0	1	1
Oral Swab	3	1	4
Oesophagoscopy Sample	0	1	1
Peritonial Fluid	1	0	1
Stool	2	1	3
Aural Swab	0	4	4
Blood	1	0	1
Drain Tip	0	1	1
Nail	1	0	1
Pus	2	1	3
Total	48	56	104

5.8.3.Biofilm formation:

5.8.3.1.Biofilm formation among *Candida* species:

Out of 104 *Candida* isolates, Biofilm formation was observed in 68 (65.3%) isolates. Biofilm production was detected in 36 out of 44 *C.tropicalis* (81.8%), 14out of 36 *C.albicans* (38.9%), 8 out of 12 *C.parapsilosis*, 5out of 7 *C.glabrata* and all the 5 C. krusei. And this difference is statistically significant with p value of .001. The details of Biofilm formation with regard to species is given in Figure 23.

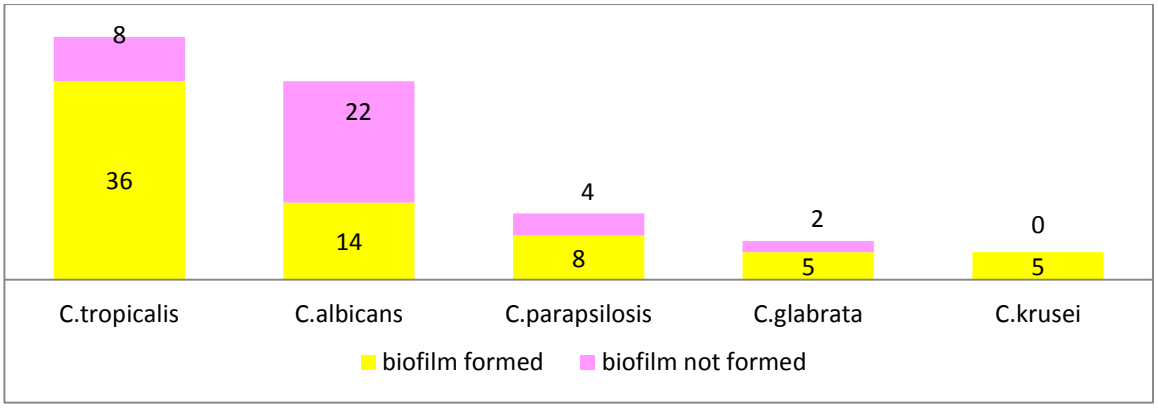


Fig 23 :Biofilm production among various *Candida* species

Most of *C.krusei* and *C.tropicalis* produced strong biofilm +++.

5.8.3.2. Association of biofilm with clinical samples:

The association of biofilm formation with regard to isolates belonging to various clinical samples is given in Table 26. In the present study, biofilm formation was demonstrated predominantly among the isolates from catheterized urine samples (32/36) followed by vaginal swab (7/9) and this difference is statistically significant with p value of < 0 .0001.

Table26: Association of biofilm with clinical samples

Samples	Biofilm formation		
	Produced	Not produced	Total
Urine Catheterised	32	4	36
Urine	3	4	7
Vaginal Swab	7	2	9
Sputum	7	22	29
Bal	2	1	3
Throat Swab	1	0	1
Oral Swab	4	0	4
Oesophagoscopy Sample	1	0	1
Peritonial Fluid	0	1	1

Stool	**2**	**1**	**3**
Aural Swab	**4**	**0**	**4**
Blood	**1**	**0**	**1**
Drain Tip	**1**	**0**	**1**
Nail	**1**	**0**	**1**
Pus	**2**	**1**	**3**
Total	**68**	**36**	**104**

5.8.3.3. Association of biofilm with catheterized and non catheterized urine samples:

Distribution of biofilm formation in relation to catheterization is shown in Figure 24 and this difference is statistically significant with p value of .004.

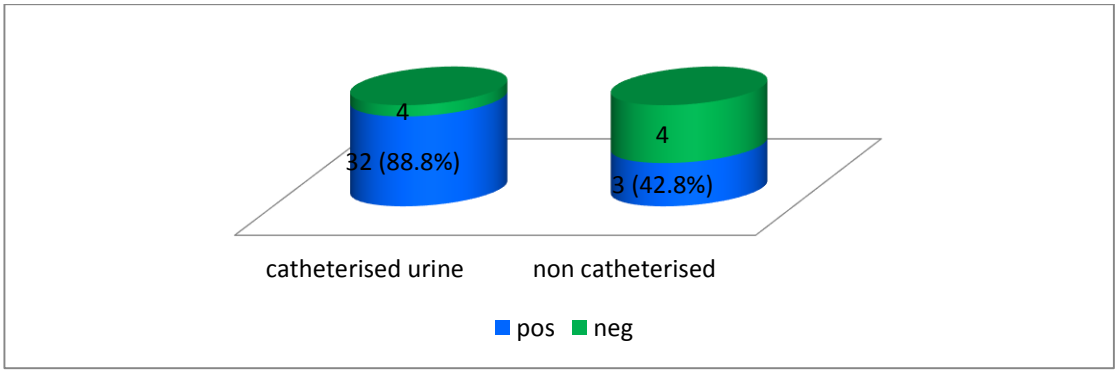


Fig 24 :Association of biofilm between catheterized and non catheterized urine samples

5.8.4.Coagulase activity:

5.8.4.1Coagulase activity among species:

Out of 104 *Candida* isolates, coagulase production was demonstrated in 26 (25%) isolates. Coagulase production was detected in 12out of 44 *C.tropicalis* (27.3%), 10 out of 36 *C.albicans* (27.8%), 2 out of 12 *C.parapsilosis* and 2 out of 5 C. krusei.

The details of coagulase activity with regard to species is given in Figure 25.

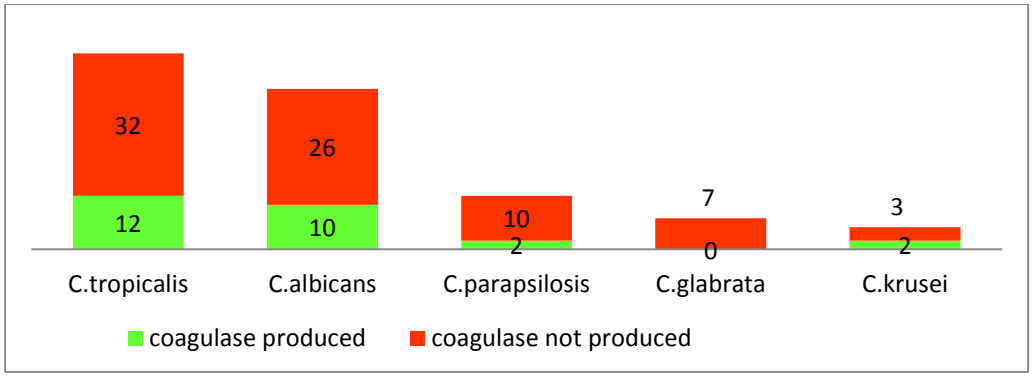


Fig 25: Coagulase production among various *Candida* species

5.8.4. Association of coagulase production among clinical samples:

The association of coagulase production with regard to isolates belonging to various clinical samples is given in Table 27. In our study the coagulase production was demonstrated in 9 out of 36 catheterised urine sample isolates, 6 out of 29 sputum sample and from the only blood isolate.This difference is statistically significant with p value of <0.0001.

Fig 27:Association of coagulase production with the clinical samples

Samples	Coagulase production		
	Produced	Not produced	Total
Urine Catheterised	9	27	36
Urine	2	5	7
Vaginal Swab	0	9	9
Sputum	6	23	29
Bal	1	2	3
Throat Swab	1	0	1
Oral Swab	1	3	4
Oesophagoscopy Sample	0	1	1
Peritonial Fluid	1	0	1
Stool	1	2	3
Aural Swab	1	3	4

Blood	**1**	**0**	**1**
Drain Tip	**0**	**1**	**1**
Nail	**0**	**1**	**1**
Pus	**2**	**1**	**3**
Total	**26**	**78**	**104**

Summation of Isolates and virulence factors:

The summary of various virulence factors in relation to *Candida* species is depicted in Figure 26.

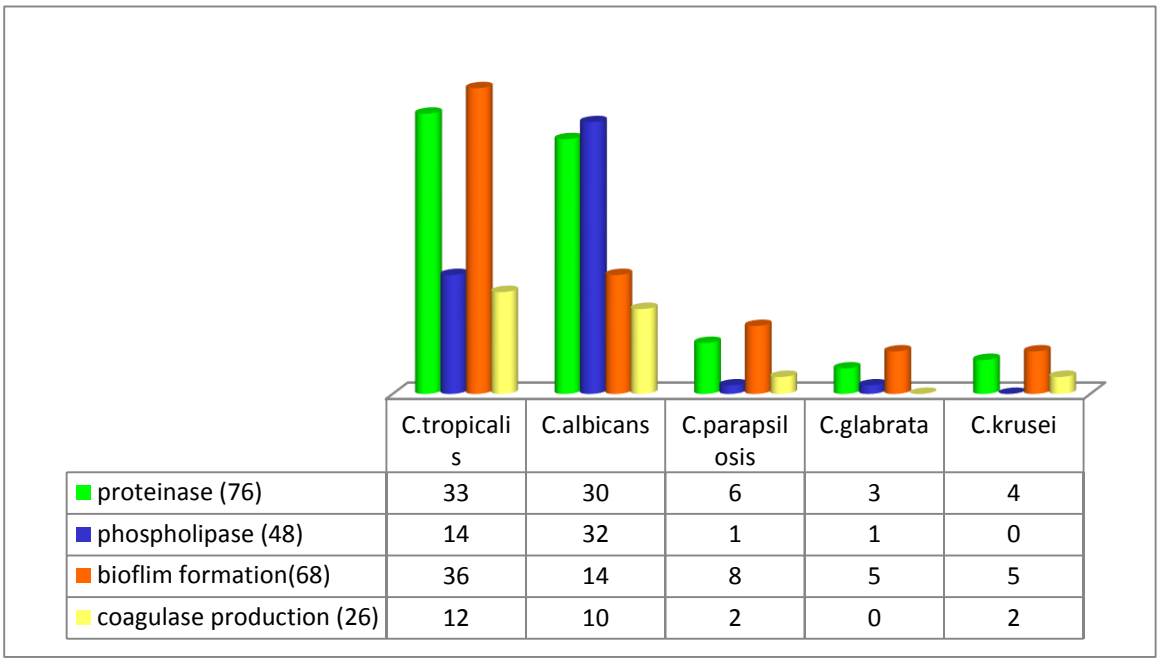
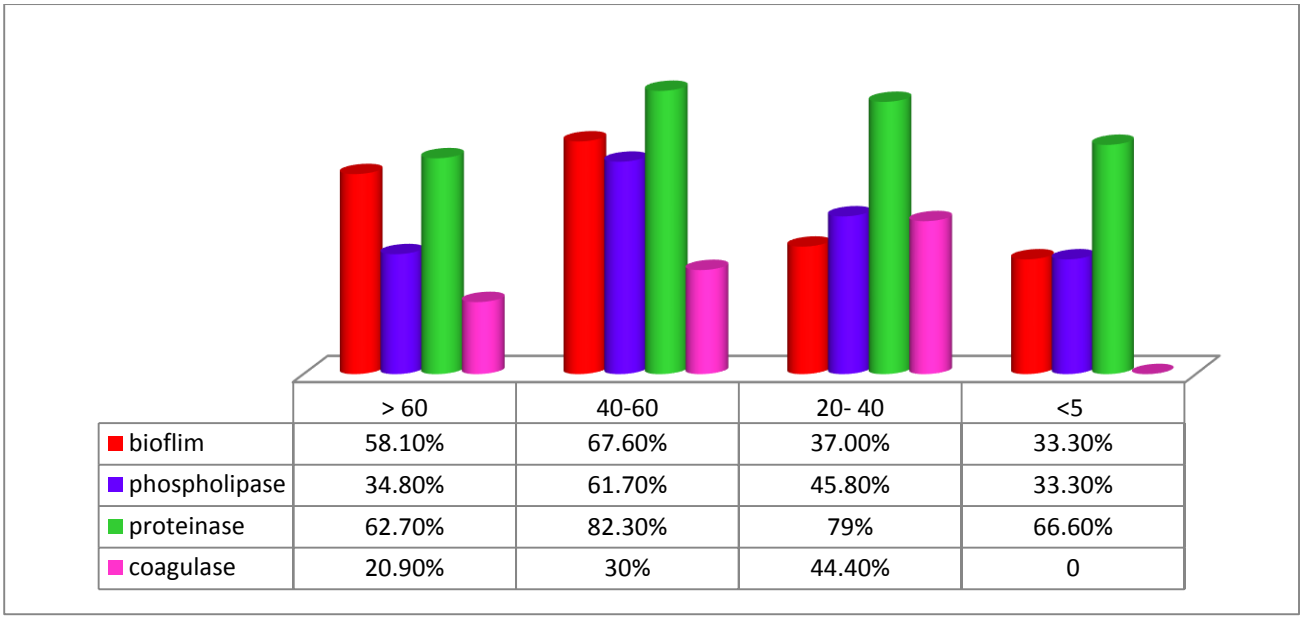


Fig 26 :Virulence factor distribution among various species

5.8.5.Distribution of virulence factor among different age groups:

The distribution of virulence factors among different age groups is depicted in Figure 27. In our study, proteinase production (82%) , phospholipase production (62%) and biofilm formation (68%) were observed commonly among 40-60 yrs of age, whereas coagulase production (44%) among 20 - 40 yrs of age. However the age of the individual is unlikely to influence the virulence factor production.

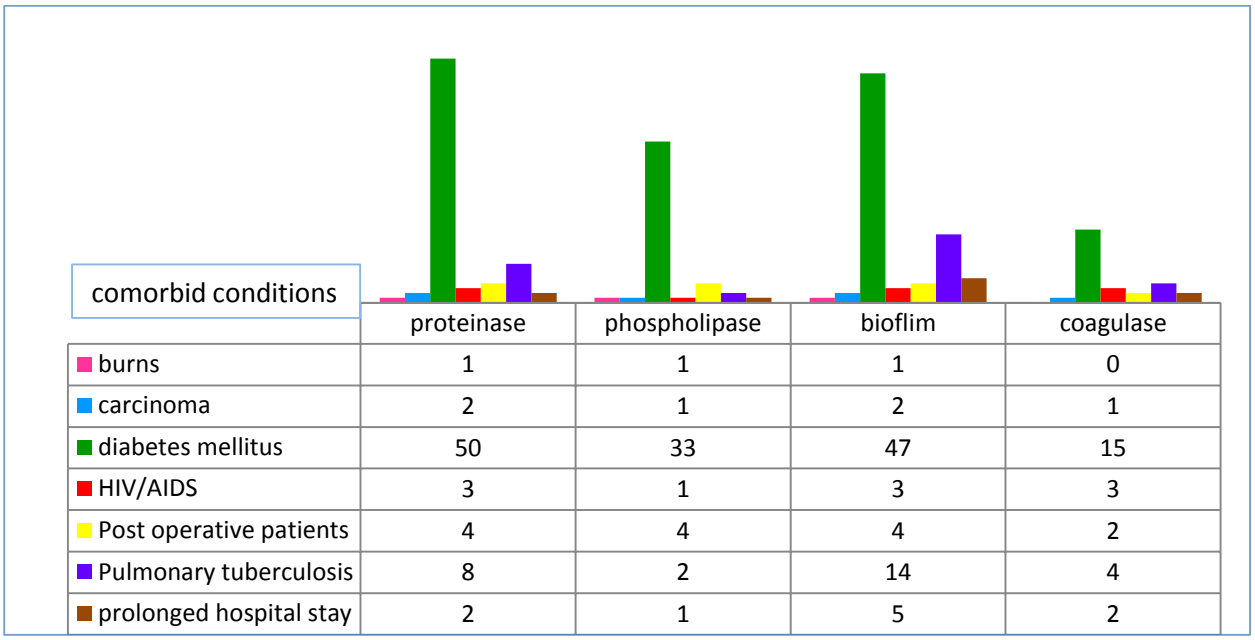


	> 60	40-60	20- 40	<5
bioflim	58.10%	67.60%	37.00%	33.30%
phospholipase	34.80%	61.70%	45.80%	33.30%
proteinase	62.70%	82.30%	79%	66.60%
coagulase	20.90%	30%	44.40%	0

Fig 27: Distribution of virulence factor in different age groups

5.8.6. Distribution of virulence factors and co-morbid conditions:

The distribution of virulence factors in relation to the co-morbid conditions is depicted in Figure 28**.** Out of 104 isolates, all the virulence factors were common among the diabetics followed by pulmonary tuberculosis patients.



comorbid conditions	proteinase	phospholipase	bioflim	coagulase
burns	1	1	1	0
carcinoma	2	1	2	1
diabetes mellitus	50	33	47	15
HIV/AIDS	3	1	3	3
Post operative patients	4	4	4	2
Pulmonary tuberculosis	8	2	14	4
prolonged hospital stay	2	1	5	2

Fig 28 : Distribution of virulence factors in various co morbid conditions

5.9.Antifungal susceptibility testing:

Antifungal susceptibility testing was carried out for all 104 isolates. Overall sensitivity of various species to fluconazole, ketaconazole and itraconazole was 75%, 31% and 60% respectively and the details are shown in the Table 28.

Table 28 : Susceptibility pattern of *Candida* species

Name and number tested	Amphotericin B		Voriconazole		Fluconazole		Ketoconazole		Itraconazole	
	S	R	S	R	S	R	S	R	S	R
C.tropicalis **(44)**	**44 (100%)**	**0**	**44 (100%)**	**0**	**31 (70.4%)**	**13**	**13 (29.5%)**	**31**	**25 (56.8%)**	**19**
C.albicans **(36)**	**36 (100%)**	**0**	**36 (100%)**	**0**	**27 (75%)**	**9**	**11 (30.5%)**	**25**	**26 (72.2%)**	**10**
C.parapsilosis **(12)**	**12 (100%)**	**0**	**12 (100%)**	**0**	**11 (91.6%)**	**1**	**4 (33.3%)**	**8**	**6 (50%)**	**6**
C.krusei **(5)**	**5 (100%)**	**0**	**5 (100%)**	**0**	**3 (60%)**	**2**	**1 (20%)**	**4**	**0**	**5**
C.glabrata **(7)**	**7 (100%)**	**0**	**7 (100%)**	**0**	**6 (85.7%)**	**1**	**4 (57.1%)**	**3**	**6 (85.7%)**	**1**

5.9.1.Susceptibility pattern of *C.tropicalis*:

The susceptibility pattern of *C.tropicalis* is depicted in the Figure 29. All the 44 *C.tropicalis* were sensitive to amphotericin B and voriconazole, 31 (70%) were sensitive to fluconazole, 13 (30%) to ketaconazole , and 25 (56%) to itraconazole.

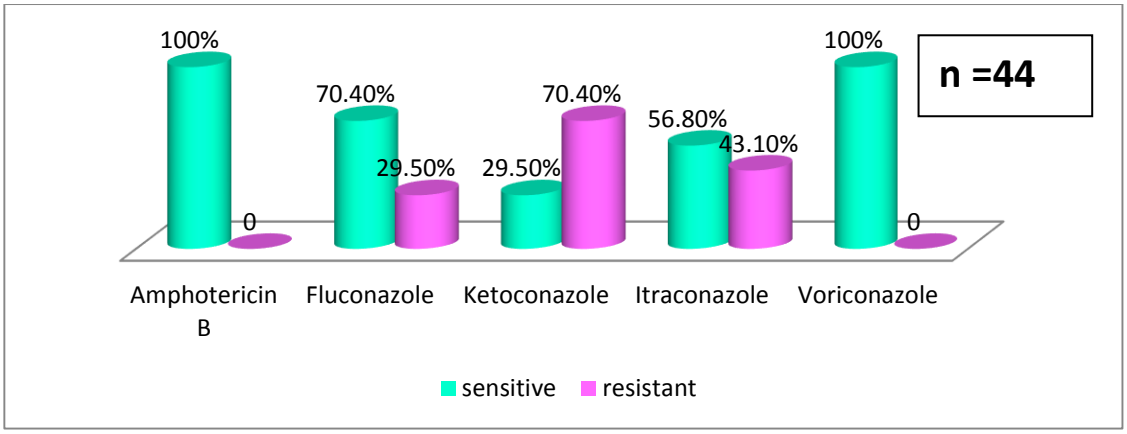


Fig 29: Susceptibility pattern of *C.tropicalis*

5.9.2. Susceptibility pattern of *C.albicans*

The susceptibility pattern of *C.albicans* is depicted in the Figure 30. Out of 36 *C.abicans* all were sensitive to amphotericin B and voriconazole. 27 (75%) to fluconazole, 11 (31%) to ketaconazole and 26 (72%) to itraconazole.

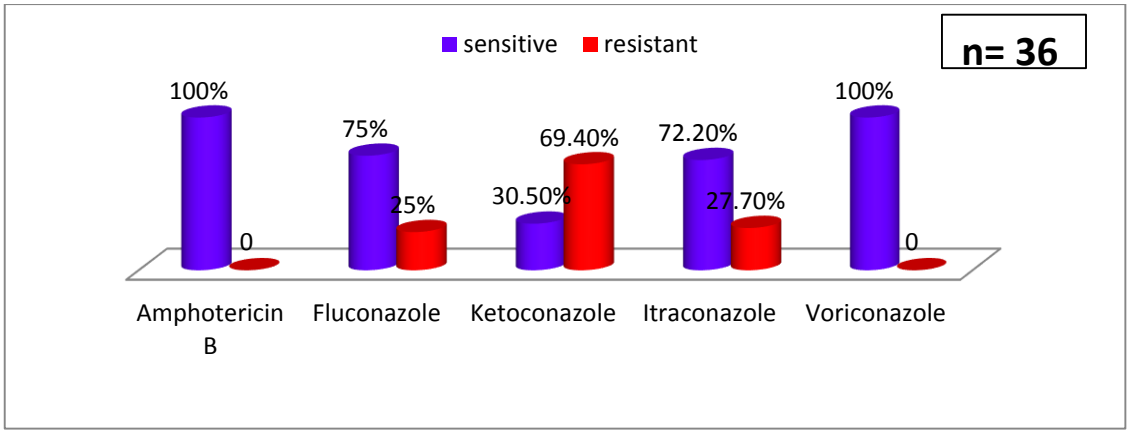


Fig 30: Susceptibility pattern of *C.albicans*

5.7.3.Susceptibility pattern of *C. parapsilosis:*

The susceptibility pattern of *C.parapsilosis* is depicted in the Figure 31. Out of 12 *C.parapsilosis* , all were sensitive to amphotericin B and voriconazole. 11 (91.6%) were sensitive to fluconazole, 4 (33.3%) sensitive to ketaconazole and 6 (50%) sensitive to itraconazole.

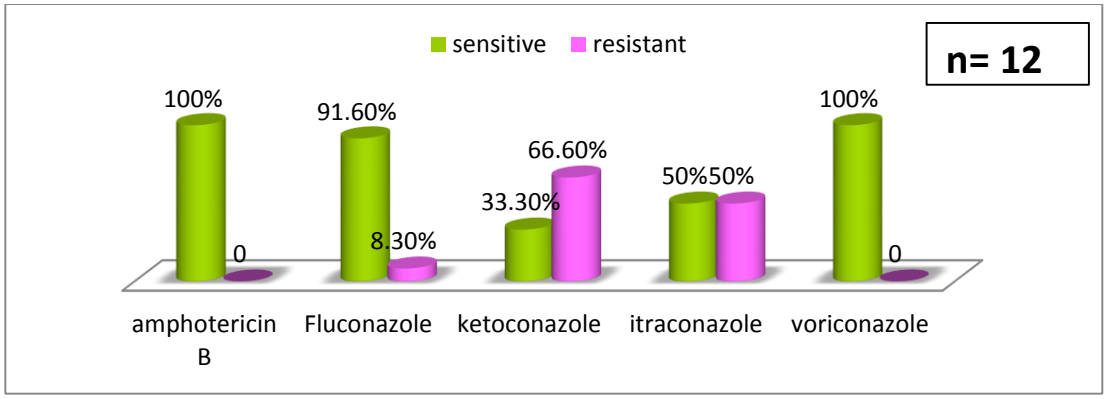


Fig 31: Susceptibility pattern of *C.parapsilosis*

5.9.4. Susceptibility pattern of *C. krusei*:

The susceptibility pattern of *C.krusei* is depicted in the Figure 32. All the 5 were sensitive to amphotericin B and voriconazole. 3(60%) to fluconazole, 1 (20%) to ketaconazole and all the 5 were resistant to itraconazole.

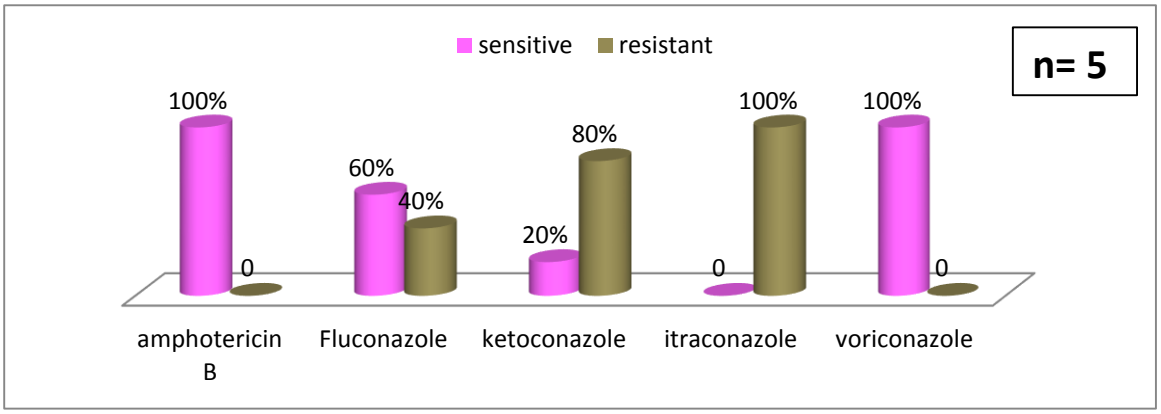


Fig 32: Susceptibility pattern of *C.krusei*

5.9.5. Susceptibility pattern of *C.glabrata*:

The susceptibility pattern of *C.glabrata* is depicted in the Figure 33. All the 7 were sensitive to amphotericin B and voriconazole. **6 (**86%) to fluconazole, 4 (57%) to ketaconazole and 6 (85%) to itraconazole.

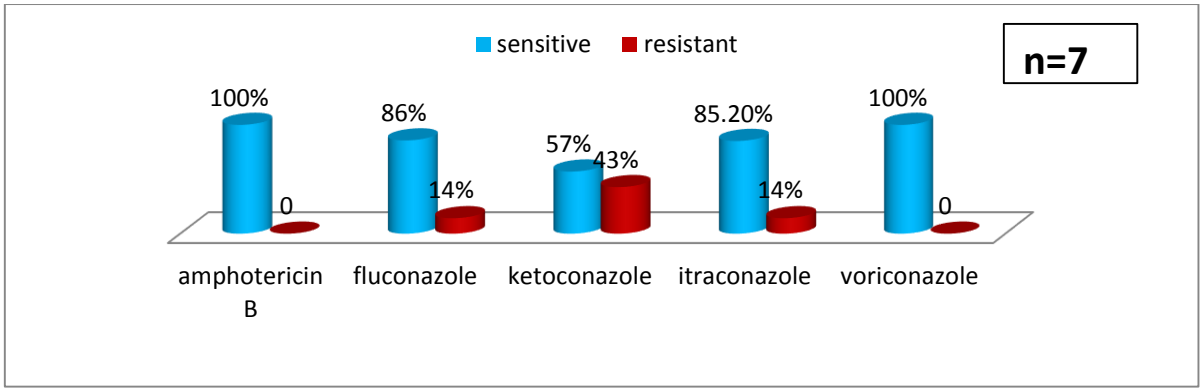


Fig 33: Susceptibility pattern of *C.glabrata*

5.9.6. Association of Virulence factor and their antifungal resistance pattern:

The distribution of virulence factor with antifungal resistance is furnished in Figure 34.

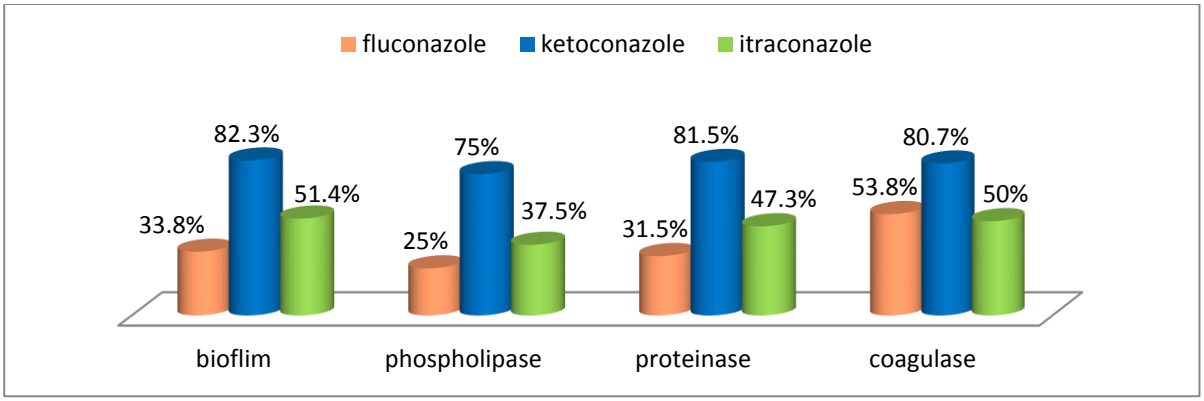


Fig 34: Distribution of virulence factors and antifungal resistance

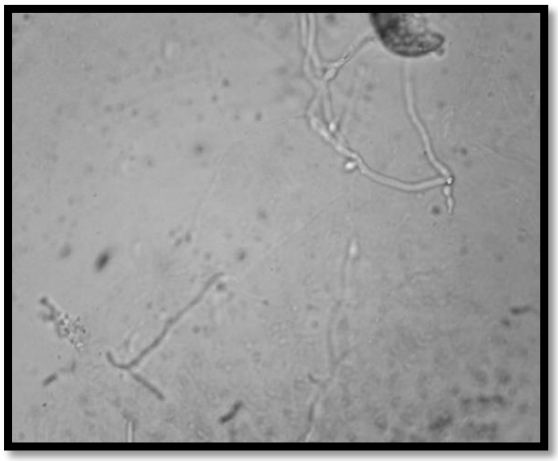
In present study, irrespective of the nature of virulence factors and species, the strains were more resistant to ketaconazole, followed by itraconazole and fluconazole and this difference is statistically significant with p value of <0.0001.

However, sub analysis between the antifungal sensitivity and the virulence factor production in relation to species of the organism did not reveal any statistical difference among each other.

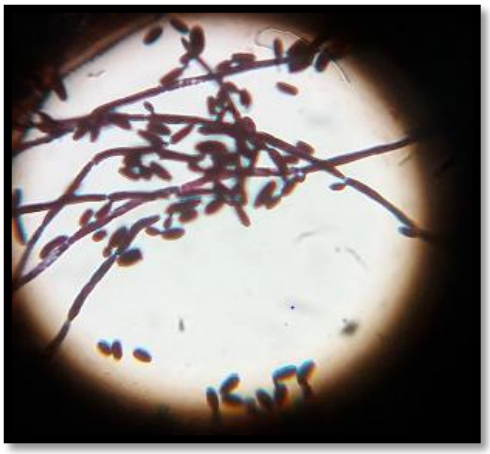
COLOUR PLATES

COLOUR PLATES

Candida isolation

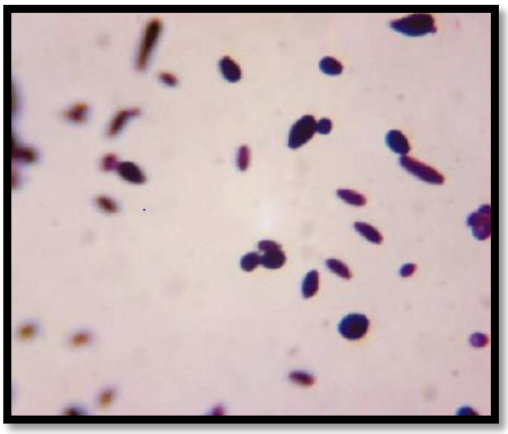


Urine Wet mount – budding yeast cells with pseudohyphae



Direct smear – Gram positive budding yeast cell with poseudohyphae

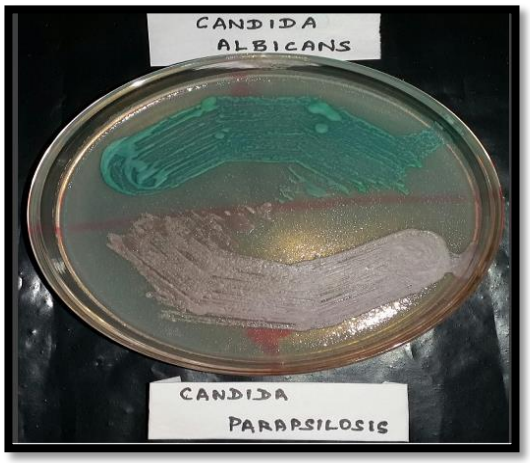
Creamy white colonies – SDA slant



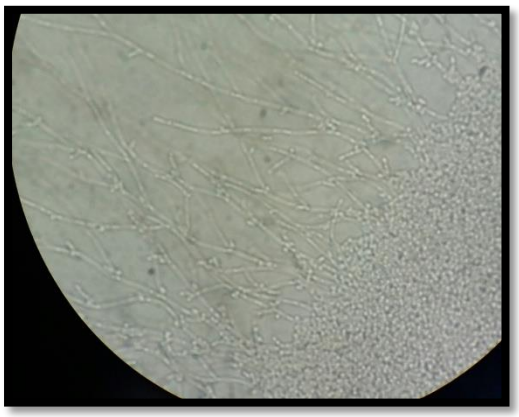
Grams stain – Gram positive budding yeast cells

Candida Speciation

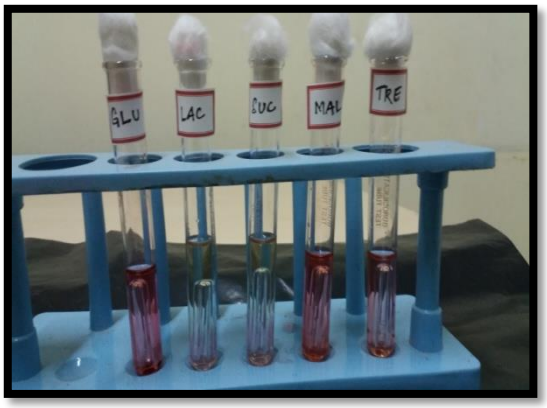
Germ Tube Test



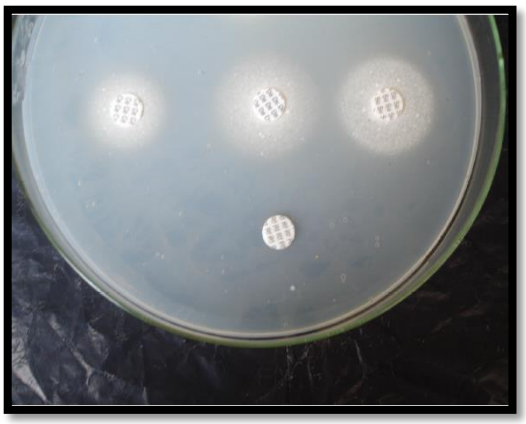
Candida CHROM agar



Dalmau technique - Chlamydospores

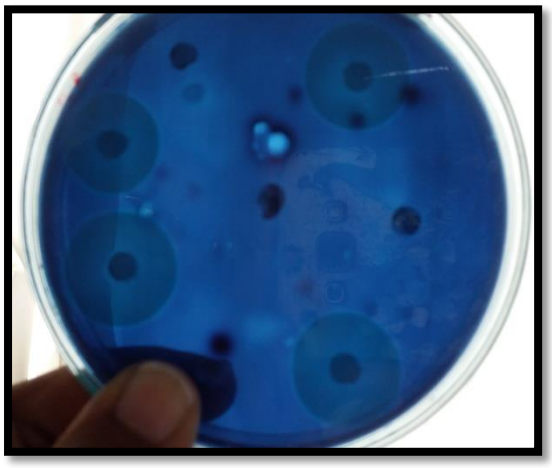


Sugar fermentation test – C. albicans

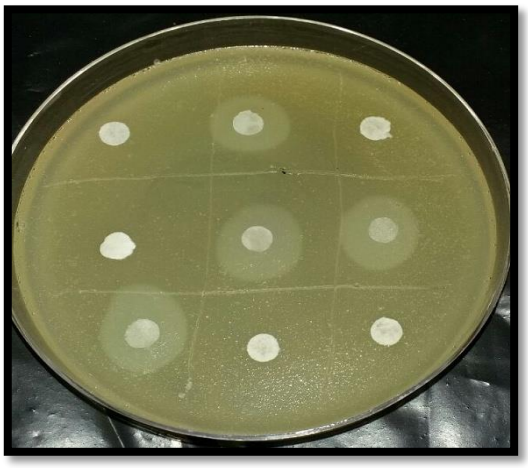


Sugar assimilation test

Candida – virulence factors detection

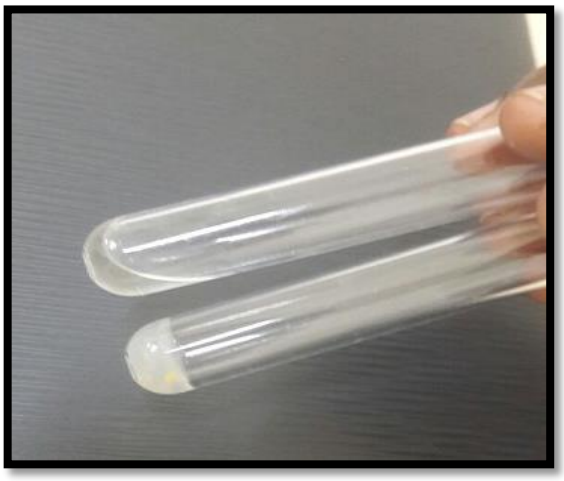


Acid proteinase production



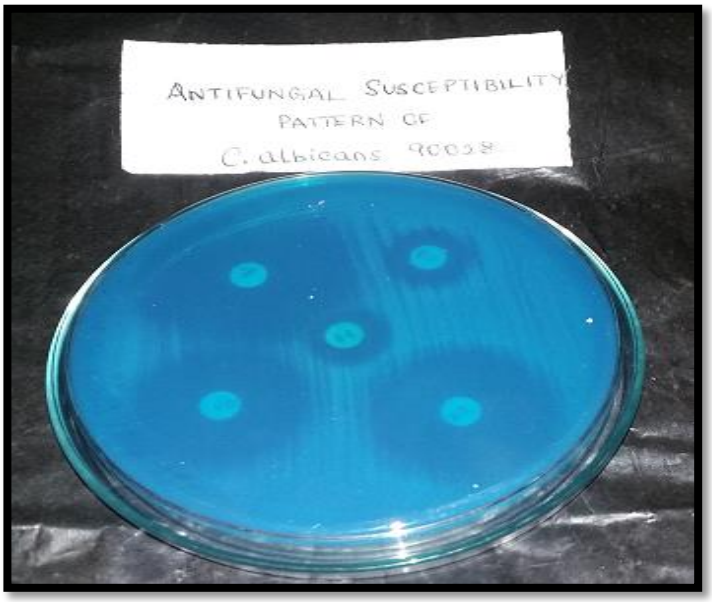
Phospholipase production – egg yolk agar

Bioflim formation - test tube method



Coagulase production

Candida – Antifungal susceptibility testing



Antifungal susceptibility testing – Disc diffusion method

DISCUSSION

6.0.DISCUSSION

Candida is an asexual, diploid fungus that is present on humans and in the environment[89]. Only a few number of *Candida* species are human pathogen. Candidial infections may vary from superficial to deep mycoses. *Candida* act as pathogens only when there is an interruption of normal host defenses.

Over the last two decades, fungal infections have increased at an alarming rate. Candidiasis has emerged as a very important opportunistic infection. In the last 20 years with the surge of *non albicans Candida species*, Clinical importance of species level identification is important as they differ in expression of virulence factors and antifungal susceptibility.

The present study was undertaken to speciate the *Candida* isolated from heterogenous clinical samples, to detect the virulence factors and to find out their antifungal susceptibility pattern. The study also concentrated on the changes observed in species distribution, the shift towards *non albicans Candida* species. in our hospital.

A total of 7,427clinical samples were received in Microbiology Laboratory during the period of February 2015 to May 2016 for culture and sensitivity which included urine (36%), pus (25 %), sputum (21 %), blood (12%), body fluids (4%).

Out of these 7,427samples, 5041(68%) samples yielded significant growth. Out of these, *Candida* constituted 104(2.05%), and the others were bacterial isolates.

The samples from which *Candida* species were isolated, constituted the study material.

6.1. Agewise distribution

In our study, the mean age was 51.75 with a standard deviation of 18.172 years. Candidiasis occurred in all age groups. This is concordant with the study conducted by Jha et al[90]. The youngest patient being two and the oldest was 85years.Isolation above 60 years was more and significant statistically ($p < 0.001$)

The age group above 60 years accounted for 41% which is the highest percentage. The aging population is especially susceptible to fungal infection mainly due to underlying medical conditions and the associated increase in the rates of hospitalization. This is concurrent with a large European based study conducted by Aikaterini Flevari et al , in which 28% of candidemic episodes were diagnosed in subjects over the age of 65 years [91]**.** These are also comparable to other studies done by Jha et al [90]. But this is discordant with the study conducted by Seshu Kumari et al where 40 -60 age group were common[92].

The second highest percentage age group is 40-60(33%) followed by 20-40(23%) and the lowest percentage (3%) was seen below 5 years of age.

6.2. Genderwise distribution

In our study, out of 104 *Candida* isolates, 53 (51%) were from male patients and 51 (49%) from female patients. There was no statistical difference with regards to gender. This is in concordance with the study conducted by Jha et al [90]. According to Murray et al. this might be attributed to more exposure of males to environment and their habit of using some addictive substances [93]. But in other studies conducted by Hidalgo et al, it is reported that colonization with *Candida* species occurred in equal numbers in both males and females [94].

6.3. Sample wise distribution of *Candida*

In our study, out of 104 *Candida* isolates majority were isolated from Catheterized urine samples 36 (34.6%) followed by sputum 29 (27.8%) and vaginal swab 9 (8.6%).This is in agreement with the study by Binesh et al [95], who stated that out of 127 *Candida* isolates 69 were from catheterized urine samples. This may be due to change in the normal flora of the perineum allowing the overgrowth of the yeast [96]. The surface of catheters also aid in colonization with *non albicans Candida species* . This is discordant with study conducted by Parvez et al where common isolate is from blood (21%) followed by urine (12%)[97].

The second most common sample is sputum (27.8%), this is similar to the report by Rakesh et al [98.]

Ward wise distribution of *Candida* isolates:

It was observed that out of 104 *Candida* isolates, 92 of *Candida* were isolated from the medical wards and the remaining from surgical wards. Out of these 92 *Candida* isolates from medical wards, maximum number of samples for *Candida* isolation were received from medicine ward 32 (31%) followed by chest and TB ward 22 (21%) and 19 (18%) from IMCU. This is discordant with the study conducted by Fadda et al [99] where the isolates were common from chest and TB ward. In other studies conducted by Ali Zarei et al [100] and Raminder Sandh et al[101] the isolates were common from IMCU.

This difference is significant statistically ($p < 0.001$)

6.4.Association between Isolates and co morbid conditions:

In our study, out of 104 *Candida* isolates, 71 (68%) were from patients

suffering from diabetes mellitus as co-morbid condition.

Table 29 : Candidiasis in diabetics – in various researches		
S.No	**Literatures**	**% associated**
1.	Madumathi et al [102]	28%
2.	Shivanand Dharwad and Saldanha Dominic[103]	32%
3.	Amar C. Sajjan et al [104]	33%

This is probably because Indians are more prone to diabetes. India is termed as the "diabetes capital of the world". According to the Diabetes Atlas 2006 published by the International Diabetes Federation, the number of people with diabetes in India is currently around 40.9 million and is expected to rise to 69.9 million by 2025. This may be due to "Asian Indian Phenotype", which refers to certain unique clinical and biochemical abnormalities in Indians which includes increased insulin resistance, greater abdominal adiposity, lower adiponectin and higher C-Reactive Protein levels [105].

Experimental evidence in vitro shows that a glucose concentration of 150mg/100ml increases the growth of *Candida* and an increase in the concentration of glucose in the tissues, blood and urine promotes the growth of *Candida*

The second most common co morbid condition is pulmonary tuberculosis (13.4%). This is concordant with the study done by Arunava Kali et al [106].

6.5. Tests for speciation:

Candida speciation was done by conventional methods which included GTT, growth at 37^0C & 42^0C, Dalmau technique, CHROM agar, sugar fermentation and sugar assimilation.

In our study, out of 104 *Candida* isolates, 44 were identified as *C.tropicalis* by Dalmau technique, CHROM agar, sugar fermentation and sugar assimilation.

Thirty six were identified as *C.albicans* by GTT, growth at both 37^0C & 42^0C, Dalmau technique and sugar fermentation and sugar assimilation. In CHROM agar, 34 produced light green colour while 2 isolates produced dark green colour.

Twelve were identified as *C.parapsilosis* by all the 4 conventional methods, 7 as *C.glabrata* and 5 as *C.krusei* by all the 4 conventional methods.

Since the Dalmau technique, sugar fermentation and sugar assimilation were concordant, these were considered as gold standard and were considered for calculating the sensitivity for CHROM agar. This is concordant with the study done by Shymala et al[107] .The other statistical screening tests like specificity, positive predictive value and negative predictive value cannot be calculated as there is no true negative values.

The sensitivity of CHROM agar for *Candida* albicans is 97.1% and this is concordant with the study done by Uma Chaudhary showing 98% for *C.albicans* [108]. Varying sensitivities have been reported by several authors for identification of *C.albicans.* Baradkar *et al.*[109] stated sensitivity of 96.55%, Willinger and Manafi [110] 98.8%, Peng *et al*[111] as 100% and Yucesoy *et al.*[112] reported 99.4% sensitivity.

In our study, two species of *C.albicans* produced dark green colour instead light green but these species grew at both 37^0C & 42^0C. Differentiation between light green and dark green colored colonies for the identification of *C.albicans* posed a problem and was found to be subjective.

Some authors described that *C.albicans* colony can produce a variation of green color, depending on the growth density and incubation period [113]. In situations here CHROM agar is used for differentiation between *C.albicans* and *C.dubliniensis,* the difference in intensity of color of colonies of these two species is not very reliable and may be lost on freezing the sample[113]

Other tests like germ tube test, growth at 42-45°C, and chlamydospore formation on CMA and Sugar assimilation test need to be done in case of ambiguity[107]

Candida tropicalis isolates produced metallic blue color on CHROM agar. Sensitivity of CHROM agar for *Candida tropicalis* was 100 %. Similar results have been reported in study done by Sumitra Devi et al[114]. These results were comparable with the study by V.P.Baradkar et al who observed a sensitivity of 100% for *C.tropicalis,* 80% for *C.parapsilosis*[109].

The germ tube test has an advantage of being simple and efficient in rapid identification of *Candida albicans* by Campbell et al [115]. But some results are liable to subjective interpretation, which makes it necessary to do repeated testing. Furthermore discrimination between germ tube and pseudohyphae needs experience.

6.5.1. Species distribution:

The present study showed that among 104 *Candida* isolates, there is predominance of *non albicans Candida species* contributing to 65% of isolates and *C.albicans* contributing only to 35% of the isolate. This was comparable to the results obtained by Manisha jain et al[116] who reported *non albicans Candida species* as predominant isolate (71.4%) from urine .

The species distribution in our study is as follows, *C.tropicalis 44* (42.3%) , *C.albicans* 36 (34.3%) , *C.parapsilosis 12* (11.5%), *C.glabrata* 7 (6.7%) and *C.krusei 5* (4.8%) . This was concordant with the study by Manisha Jain et al [116] in north India. Manisha Jain et al observed that 52.9% of isolates were *C.tropicalis* and 29.8% were *C.albicans*.

Isolation and speciation done by various workers:

Table 30: Speciation of *Candida* by various workers

Species of *Candida*	**Our study**	**Shiva prakash[117] 2007**	**Capoor[118] 2007**	**Lymn[119] 2003**	**Prasad[120] 1999**	**Baradkar[121] 1999**	**Pfeller[122] 1999**	**Frank[123] 1994**
C.tropicalis	**42.3**	**35.6**	**38.7**	**24**	**35.4**	**17.94**	**11**	**7.43**
C.albicans	**34.3**	**3.4**	**25.8**	**24**	**47.6**	**76.92**	**52**	**39.2**
C.parapsilosis	**11.5**	**28.8**	**25.8**	**6**	**1.2**	**-**	**-**	**10.1**
C.krusei	**4.8**	**-**	**6.4**	**10**	**4.9**	**-**	**2**	**5.9**
All figures mentioned above are expressed as percentage								

6.5.2. Distribution of *Candida* species among different age groups:

In our study, out of 104 *Candida* isolates, C. glabrata is more common in age group more than 60yrs (57.10%). This is concordant with the study conducted by John Paul et al, who reported that *C. glabrata* accounted for more than 18% of isolates in women older than 51 yrs compared to 4.8% for young adults [124]. This increase in non-*albicans* species, specifically *C. glabrata*, is an important observation that was reported in smaller studies of the elderly [124]. Higher prevalences of non-*albicans* species in menopausal and postmenopausal women may be explained due to the changes in patient physiology, hormone balance, and decrease in immune function[124].

C.krusei is common among 40 – 60 yrs of age (40%) followed by *C.albicans* (38.9%). This is concordant with the study conducted by Francisca et al[125], who reported that *C.albicans* (33.3%) was the dominant species followed by *C.glabrata* (16.7%) and *C. tropicalis* (16.7%).

C. parapsilosis is common among 20 – 40yrs of age (33.3%). Significant increase in the prevalence of C. parapsilosis has been studied by John Paul et al [124]. *C.albicans* is common among children less than 5 yrs (5.6%). This is in accordance with the study by John Paul et al, who stated that *C.albicans* is common in less than 20 years of age [124].

6.5.3. Distribution of *Candida* species among heterogenous clinical samples:

Out of 104 *Candida* isolates**,** *C.tropicalis* were commonly isolated mainly from respiratory specimen, catheterized urine, and vaginal swab. This is concordant with the study done by Sumithra devi et al, who stated that *C.tropicalis* was commonly isolated from catheterized urine samples [114]. *C.albicans* were commonly isolated from respiratory specimens, urine and blood. This is in agreement with the study done by Mohandas Vinitha et al [89]. In our study *C.glabrata* were isolated from sputum and urine. *C.parapsilosis* from respiratory samples and urine, *C.krusei* from catheterized urine samples. This confers to the report by Mohandas Vinitha et al [89].

6.6. Virulence factors:

6.6.1. Proteinase activity in relation to clinical samples and species of *Candida*:

Proteinase enzymes are secreted by pathogenic C*andida* species during infection. They are responsible for invasion of host response, adhesion and tissue

damage. The cultivation of *Candida* in Yeast Carbon Base-Bovine Serum Albumin at acidic pH, induces the secretion of Saps.

In our study, out of 104 *Candida* isolates, 76 (73%) produced acid proteinase. This is consistent with the study conducted by Kantarcioglu and Yucel et al who observed that 78.9% of all *Candida* strains were proteinase-positive [126].

In our study the Proteinase production was detected in 30 (83.3%) *C.albicans* isolates. This is accordant with the study by Kantarcioglu and Yucel et al who reported that 95% of *C.albicans* produced proteinases [126].

In our study, the proteinase activity was seen among 30 out of 36 catheterized urine samples (83.3%), isolates from all the 9 vaginal swab (100%) and one isolate from blood and this difference is statistically significant with p value of < 0.0001.

This is confirmable with the study by Kantarcioglu et al, who reported that isolates from 91.3% of urogenital samples were proteinase positive[126]. Another study done by Livia de Souza et al is also concordant with our study, and he reported that Proteinase activity was found more in blood isolates (81.2%), followed by urine samples (78.1%) [127]. This association of proteinase activity with the clinical sample is statistically significant with p value of < 0.0001.

Thirty three (75%) of *C.tropicalis* produced proteinase activity this is consistent with the study by Yamamoto et al who reported 65–75% of C. tropicalis with secreted aspartic proteases activity [128].

The extracellular proteinases produced by C. albicans is the most important virulence factor. Decreased expression of the enzymes indicates decreased virulent

nature of *Candida* species and the degree of virulence and pathogenicity are correlated with the level of secreted proteinases [129].

6.6.2.Phospholipase activity in relation to clinical samples and species of *Candida*:

Phospholipase enzymes are important virulence factor and these are associated with membrane damage of the host cells, adherence, and penetration. Invasion of host cells by microbes entails penetration and damage of the outer cell envelope. Early data suggest that direct host cell damage and lysis are the main mechanisms contributing to fungal virulence. Phospholipase production may be used as one of the parameters to distinguish virulent invasive strains from non-invasive colonizers.

In our study out of 104 *Candida* isolates 48 (46%) produced phospholipase. This confirms with the study conducted by Deepa et al, in which it is reported that phosholipase production was observed among 52.6% of isolates [130].

Phospholipase production was detected in 32 (89.9%) *C.albicans*. This is consistent with the study done by Sachin et al and it is reported that 92.3% of *C.albicans* produced phospholipase [131].

In our study, the phospholipase production among *Candida non albicans* is more commonly observed among 43.3 % of *C.glabrata*, followed by *C.tropicalis* (31.8%) and *C.parapsilosis* (8.3 %) . But this is discordant with the study conducted by Sachin et al, who reported that among the *Candida non albicans* species, the maximum phospholipase production (76%) was observed in *C.tropicalis* [131]. Other study done by Thangam et al. is also discordant with our study who reported high phospholipase activity in *C.tropicalis* [132].

And in our study, interspecies phospholipase activity is statistically significant with p value of < 0.0001.

The phospholipase production was more among the isolates from pus (55.5), followed by vaginal swabs (55%) and catheterized urine samples (44%). This is disconcordant with the study done by Ruchika et al, who reported that Phospholipase producers were predominently from high vaginal swab (63.2%), followed by pus 3 out of 7 (42.9%), urine 18 out of 49 (36.7%) [133]and the association between phosholipase production and clinical sample is statistically significant at p value of < 0.0001.

6.6.3.Biofilm formation in relation to clinical samples and species of *Candida*:

Biofilms are a collection of microorganisms surrounded by the slime they secrete. The ability to form biofilms is associated with the pathogenicity and as such should be considered as an important virulence determinant during candidiasis[134]. Most often fungi form biofilm on surface of device used in clinical practices such as intravascular catheters, dentures, heart valves, implanted devices and contact lenses. They are indeed difficult to eradicate by host immune mechanism and the associated organisms are highly refractile to antimicrobials [135].

In our study, out of 104 *Candida* isolates, Biofilm formation was detected in 68 (65.3%) isolates. This is concordant with the study conducted by Saroj Golia et al. who reported that 65.74% of the *Candida* isolates tested were found to be biofilm producers[136]. It is also consistent with the study conducted by Muni et al (64%) [134].

In our study, biofilm formation was more common among *Candida* non *albicans* (61.1%) than *Candida albicans* (38.9%). This is in agreement with the study conducted by Muni et al[134].

Among the *Candida non albicans*, biofilm production was detected in all the 5 isolates of C. krusei (100%), 36 (81.8%) *C.tropicalis*, 5 (71.4%) *C.glabrata* and 8 (66.7%) of *C.parapsilosis*. This is accordant with the study conducted by Saroj Golia et al, who observed that among the *Candida non albicans* species, the biofilm formation was common among isolates of *C.krusei* (80.77%), followed by *C.tropicalis* (72.73%) and *C.parapsilosis* (50%) [136]. Most of *C.krusei* and *C.tropicalis* produced strong biofilm +++. This is concordant with the study conducted by Mohandas et al. It has been recognized that *C.krusei* and *C.tropicalis* are strong slime producers by many studies [89] and the interspecies biofilm formation is statistically significant at p value of .001.

In our study, biofilm formation was more common among the isolates from catheterized urine samples (88.8%) followed by vaginal swab (77.7%). This is concurring with the study conducted by Bansal et al and it is reported that biofilm producers were common from 35 (60.9%) urine isolates followed by 7(46.1%) vaginal swabs[137]. And this difference is statistically significant at p value of < 0.0001.

In our study, out of 36 catheterised urinary isolates 32(88.8%) produced biofilm formation. On the other hand, out of 7 non catheterized urinary sample 3 produced biofilm (42.8%). This is concordant with the study conducted by Mythreyi et al[138] and this is statistically significant at p value of .004.

6.6.4.Coagulase production in relation to clinical samples and species of *Candida*:

In our study out of 104 *Candida* isolates coagulase production was detected in 26 (25%) isolates. This is concordant with the study conducted by Nimet yigit et al, who reported coagulase production in 47.3% of *Candida* isolates[139].

In our study, coagulase production was detected in 10 (27.8%) *C.albicans*, 2(40%) *C.krusei*, 2(16%) of *C.parapsilosis*, 12(27.3%) of *C.tropicalis*. This is concordant with the study conducted by Nimet Yigit et al, it is reported more coagulase activity in *Candida* albicans than *Candida non albicans*. 64.7 % of *Candida albicans* produced clot with rabbit plasma followed by *C.krusei* (42.8%) and *C.parapsilosis* (40%) [139].

6.6.5.Association of virulence factor with age groups:

In our study, biofilm formation (68%), proteinase (82%) and phospholipase production (62%) are common among 40-60 yrs of age, whereas coagulase production (44%) virulence factor is common among 20 - 40 yrs of age. Only limited studies are available regarding the association of virulence factor and age group. In a study conducted by Jin et al, stated that there is lower degree of biofilm formation in age groups older than 35 yrs compared to the biofilm formation in age group less than 35 yrs[140].

6.6.6.Association of virulence factors and co morbid conditions:

In our study, out of 104 isolates, all the virulence factors were common among the diabetic mellitus followed by pulmonary tuberculosis patients. This is concordant with the study conducted by Deepa Anil kumar et al and they reported that the proteinase and phospholipase activity of *Candida* isolates are common among the diabetics [141].

Many literatures have shown that there is a direct relationship between the ability to produce biofilm and its pathogenicity [142].

6.7.Antifungal susceptibility testing

Resistance to Azoles and polyenes continues to increase and is a matter of concern as this is the most commonly used empirical therapy for suspected fungal infections

In our study, out of 104 *Candida* isolates, all were sensitive to Amphotericin B and voriconazole. This is agreeable with the study conducted by Mokaddas et al who reported that most of their isolates were sensitive to amphotericin-B [143]

In our study, sensitivity to fluconazole is 75%. This is concordant with the study conducted by Adhikary et al, who reported that 75% of *Candida* isolates were sensitive to Fluconazole[144]. But this is discordant with the study conducted by Parvez Anwar et al who reported that most of the *Candida* isolates (32 out of 49 resistant strain) were resistant to fluconazole [145]

6.7.1**Susceptibility pattern of *C.tropicalis***

In our study, out of 44 *C.tropicalis* all were sensitive to amphotericin B and voriconazole. This is concordant with the study conducted by Binesh Lal et al who reported that all the isolates were sensitive to amphotericin B [146].

The sensitivity of *C.tropicalis* in our study is 70% to fluconazole. This is concurrent with the study conducted by Binesh Lal et al and he reported that 62% were sensitive to fluconazole[146]. But this is discordant with the study conducted by Myoken et al who reported maximum resistance to fluconazole [147.] .

In our study, *C.tropicalis* were mostly resistant to ketaconazole (70.4%). This is concordant with the study conducted by Ariane Bruder-Nascimento et al, who observed *C.tropicalis* being the most resistant to ketaconazole [148].

6.7.2.Susceptibility pattern of *C.albicans*

In our study, out of 36 *C.albicans,* all were sensitive to amphotericin B and voriconazole. This is concordant with the study conducted by Fadda et al who reported that 97.7% were sensitive to amphotericin B [149].

In our study, 75% *C.albicans* were sensitive to fluconazole and 25% were resistant. This is concurrent with the study conducted by Rizvi MW et al who reported 20% of fluconazole resistance among *C.albicans* [150]. But this is inconsistent with the study by Ariane Bruder Nascimento et al who reported high sensitivity to fluconazole [148], ketaconazole (31%) and to itraconazole (72%).

6.7.3.Susceptibility pattern of *C.parapsilosis*

In our study, all 12 isolates of *C.parapsilosis*, were sensitive to amphotericin B and voriconazole. This is in accordance with the study conducted by Meng Xiao et al, who reported 99.2% susceptibility of *C.parapsilosis* to voriconazole.[151]. In our study, 91.6% were sensitive to fluconazole, which is also in concordance with the study by Meng Xiao who reported that fluconazole were sensitive in 97.7% of isolates [151].

In our study 33.3% was sensitive to ketoconazole, this is discordant with the study conducted by Kamiar Zomorodian et al, who reported 88.5 % sensitivity to ketoconazole[152]. In our study 50% were sensitive to itraconazole and this is in discordance with the study conducted by Kamiar Zomorodian et al, who observed that 92.3% were sensitive to itraconazole[152].

6.7.5.Susceptibility pattern of *C.glabrata*

In our study, all the 7 *C.glabrata* isolates were sensitive to amphotericin B and this is concordant with the study by Kamiar Zomorodian et al who reported that all isolates were sensitive to amphotericin B [152].

In our study, all the 7 isolates were sensitive to voriconazole. This is concordant with study by Fadda et al who reported that Voriconazole was the most efficient antimycotic for *Candida glabrata* with a sensitivity of 100% [149].

In our study 86% of C. *glabrata* were sensitive to fluconazole. This was also concordant with the study by Kamiar Zomorodian et al and he reported that 90.5% were sensitive to fluconazole [152]. In our study, 57% were sensitive to ketaconazole. This is concordant with the study conducted by Kamiar Zomorodian et al, who reported that 50% were sensitive [152].

In our study 85% were sensitive to itraconazole this is inconsistent with the study by Kamiar Zomorodian et al , it is reported that 59% were sensitive to itraconazole[152].

6.7.4.Susceptibility pattern of *C.krusei*

In our study, all the five *C.krusei* isolates were sensitive to amphotericin B and voriconazole. This is agreeable with the study conducted by Fadda et al who reported that Voriconazole was the most efficient antimycotic for C. krusei with sensitivity of 96.7%[149].

In our study 3 out of 5 (60%) *C. krusei* were sensitive to fluconazole, this is discordant with the study conducted by Kamiar Zomorodian et al , it is reported that 100% were sensitive to fluconazole [152].

6.7.6.Association of Virulence factors and their resistance pattern:

In our study**,** the biofilm producing strains were more resistant to ketoconazole (82.3%) followed by itraconazole (51.4%) and fluconazole (33.8%). This is in agreement with the study by Semiha Ozkanet al who reported that Biofilm production was associated with high-level antimicrobial resistance[153].

The phospholipase producing strains were more resistant to ketoconazole (75%) followed by itraconazole (37.5%) and fluconazole (25%). In a study by Rajendra Kothavade et al, it is reported that there is a significant correlation between phospholipase activity and fluconazole resistance. Nystatin and amphotericin B, but not fluconazole, significantly reduce the phospholipase activity of both *C. albicans* and *C. tropicalis* species [154].

The proteinase producing strains are more resistant to ketoconazole (81.5%) followed by itraconazole (47.3%) and fluconazole (31.5%). This is concordant with the study conducted by Semiha Ozkanet al, who reported a positive correlation between proteinase production and antifungal susceptibility [153].

Suggestions:

Futher studies are suggested to find out an association between virulence factor production, the clinical course as well as the therapeutic response. As the co-morbid conditions have a precipitating role for the conversion of commensal state into pathogenic state, the possibility of segregating commensal from the pathogen is difficult at present and requires a detail study.

Since *Candida* is likely to be ignored by the treating doctors, it is suggested that the treating doctor if gets a report of *Candida* isolated from biological sample, the

treating doctor must be motivated to interact with the microbiologist and make decisions before administrating antifungal drugs.

SUMMARY

7.0. Summary

Fungal infections are gaining importance in clinical practices for various reasons. Among the fungal agents, *Candida* infections are frequently encountered. Hence, the present study was undertaken.

This study entitled "**Speciation, Virulence Factors Detection and Antifungal Susceptibility testing of *Candida* isolated from heterogenous clinical samples**" was carried out in the department of Microbiology, Chennai Medical College Hospital and Research Centre, Trichy from February 2015 to May 2016 by adopting CLSI guidelines, and after approval from IEC.

- During the study period, 7,427 clinical samples were received in Microbiology Laboratory, out of which significant growth was observed in 5041 (68%). Of these 5041 isolates, 104 (2%) belonged to *Candida species*.
- The youngest patient was two years old and the oldest was 85, and the mean age was 51.7years. Isolation above 60 years was more and significant statistically ($p < 0.01$).
- Gender wise 53(51%) were from males and 51(49%) from females.
- The isolates of *Candida* with regard to the clinical samples, were in the order of catheterized urine (34.6 %) followed by sputum (27.8%), vaginal swab (8.6%) and others (29 %).
- Ward wise distribution of *Candida* isolates showed that 92 (88%) were from medical wards and the remaining from surgical wards.
- *Candida* isolates were more from inpatients (82 %) than outpatients (18%).

- The co-morbid conditions noticed among the cases from whom *Candida* species were isolated includes diabetes mellitus (68%), pulmonary tuberculosis (13%), HIV (3%), carcinoma (2%), prolonged hospitalization and ICU stay (2%), burns (1%) and post operative status (1%). No co-morbid conditions was noticed in 7% of cases.
- When all the 104 *Candida* isolates were subjected to GTT and growth at both 37^0C and 42^0C, only 36 (35%) were identified as *Candida albicans* and the rest were *Candida* non albicans (n=68 ;65%).
- In our study, 44 (42.3%) were identified as *C.tropicalis*, 36 (34.6%) as *C.albicans*, 12 (11.5%) as *C.parapsilosis*, 7 (6.7%) as *C.glabrata* and 5(4.8%) *C.krusei* by Dalmau technique, CHROM agar, sugar fermentation and sugar assimilation.
- Out of 36 *C.albicans*, two of them revealed dark green colour in CHROM agar instead of light green, and when these two were subjected to growth at both 37^0C and 42^0C they revealed as *C.albicans* thus ruling out *C.dubliniensis*. This chemical versus physical method of confirmation made us to realise that the physical factors are more dependable to differentiate *C.albicans* from others *Candida species* than chemical methods, and it is also feasible, economical and time saving.
- Four virulence factors were looked for and appropriate tests were carried out for all the 104 *Candida* isolates. The virulence factor acid proteinase production was detected more 76 (73%) during the study period, followed by

biofilm production, phospholipase production and coagulase production in the order of 68 (65.3 %), 48 (46%) and 26 (25%) respectively.

- When the production of virulence factor was analysed in relation to different clinical sources, biofilm formation was more in *Candida* species isolated from catheterized urine samples, and acid proteinase was more from sputum samples. However, a definitive statistical conclusion could not be made as the isolates were small in number in relation to the source/ sample.
- The capacity to produce the virulence factor is not influenced by the underlying co-morbid conditions.
- Since most of the *Candida* isolates were obtained from patients with one or other co morbid conditions, the isolates cannot be ignored as commensal on the basis of non production of one or other virulence factors assessed during the study.
- Irrespective of the virulence factor production by the *Candida* isolates, all the 104 were sensitive to Amphotericin B and Voriconazole.
- Sensitivity of various *Candida* species to fluconazole, ketaconazole and itraconazole were 75, 31 and 60% respectively.
- A definitive conclusion could not be arrived when the resistance status of *Candida* species was analyzed in relation to different virulence factors as the numbers were small in each sub group. Hence, it is suggested to conduct similar studies with large number of samples before making any embarking statements or recommendations.

CONCLUSION

8.0. Conclusion

Among the fungus, *Candida* is the commonest noscocomial pathogen. The identification of *Candida* upto species level has a major role for therapy and surveillance.

- ✓ Out of the 7,427 clinical samples received in Microbiology Laboratory, significant growth was observed in 5041 (68%). Of these 5041 isolates, 104 (2%) belonged to *Candida species*.
- ✓ These 104 isolates were subjected to speciation by GTT and growth at 37^0C and 42^0C. Thirty six were identified as *C.albicans* which constituted 35%.
- ✓ When all the 104 isolates were screened by CHROM agar, Dalmau technique, sugar assimilation and sugar fermentation, 44 (42.3%) were identified as *C.tropicalis*, 36 (34.6%) as *C.albicans*, 12 (11.5%) as *C.parapsilosis*, 7 (6.7%) as *C.glabrata* and 5(4.8%) as *C.krusei.*
- ✓ Speciation made us to realize that, this centre has many *Candida non albicans* species which concurs with published literature.
- ✓ Most of the isolates were obtained from the patients who had one or more co-morbid conditions.
- ✓ The species of *Candida* isolated did not have any association with the age of the patient and the underlying disease or the co-morbid status.
- ✓ Production of four of the virulence factors was studied - i) acid proteinase production, ii) phospholipase production in egg yolk medium, iii)biofilm formation by test tube method and iv) coagulase production. When these 104 isolates were analysed in relation to each of them, the results revealed variable

response, i.e., 76 (73%) produced acid proteinase, 68 (65.3 %)biofilm production, 48 (46%) phospholipase production and 26 (25%) coagulase production. Since all these isolates were from sick patients with or without co-morbidities, the isolates which were negative for virulence factor production, cannot be ignored as commensal.

- ✓ The study has brought out the importance of recognizing *Candida* species as pathogen based on the clinical status.
- ✓ All the 104 isolates were sensitive to antifungal agents such as Amphotericin B and Voriconazole. Sensitivity status of all these isolates for Ketaconazole, Fluconazole and Itraconazole when correleated with the virulence factor production, age of the patient and co-morbid conditions, a definitive association could not be made with any of the parameters considered.
- ✓ These isolates have developed resistance to commonly used antifungal agents such as fluconazole, ketaconazole and itraconazole and it was 25%, 69% and 40% respectively.
- ✓ The isolation and their resistance pattern make us to consider the need for surveillance system for fungal isolates atleast in teaching institutions to begin with.

Strengths of the study:

- Good laboratory practices was adopted at all levels viz., sample collection and processing, isolation and identification, speciation and antifungal testing.
- The works were monitored by two senior faculty members independently.
- Standard media and chemicals were purchased for lab works.

Limitations of the study:

- It is a single center study.
- Molecular studies for species confirmation were not performed.
- Access to the Echinocandins sensitivity in Indian settings was not possible due to limited resources.

ANNEXURE I

ஒப்புதல் படிவம்

பங்கேற்பாளரின் பெயர் :

ஆராய்ச்சியின் தலைப்பு :

இந்த ஆராய்ச்சியை பற்றிய அனைத்து விவரங்களும் எனக்கு எழுத்து மூலமாகவும் மற்றும் வாய் மொழியாகவும் எனது தாய் மொழியில் முழுமையாக விளக்கப்பட்டது. இவ்வாராய்ச்சியை முழுமையாக புரிந்து கொண்டேன். இவ்வாராய்ச்சியை பற்றிய கேள்விகளை எழுப்புவதற்கு எனக்கு வாய்ப்பு அளிக்கப்பட்டது. இவ்வாராய்ச்சியில் நான் பங்கு பெறுவது என்னுடைய சொந்த விருப்பத்தை பொறுத்தது என்றும், இந்த ஆராய்ச்சிக்காக எனது உடலின் திரவ மாதிரிகள் [சிறு நீர், இரத்தம், கோழை (சளி)] போன்வற்றை கொடுத்து, இவ்வாராய்ச்சி செய்ய நான் மனமார சம்மதிக்கிறேன். இதற்கு நான் எந்த தடையும் இடையூறும் செய்யமாட்டேன் என்று சுயநினைவுடன் உறுதியளிக்கிறேன்.

பங்கேற்பவர் கையொப்பம் தேதி

CHENNAI MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE

"SPECIATION, VIRULENCE FACTORS DETECTION AND ANTIFUNGAL SUSCEPTIBILITY TESTING OF CANDIDA ISOLATED FROM HETEROGENOUS CLINICAL SAMPLES"

PROFORMA:

Sample no :

Name of the patient :

Age /sex :

IP/OP No :

Dept/ward :

Pre disposing factors/

coexisting condition: Pregnancy/TB/Sepsis/DM/Prolonged Contact With Water

Treatment history : Antibiotics/Steroids/ Immunosuppressants/OCP

Any procedure done : Catheterised/Ventilator/IUCD

Hospital stay : D.O.A- D.O.D-

Patient outcome : Improved/Cured/Expired/AMA

Diagnosis :

LAB DETAILS:

Sample collected:

Investigations

Direct smear/ wet mount – pseudohyphae/ budding yeast cells

Growth on NA /BA

ISOLATION	Gram staining	
	Culture in SDA	
Speciation	GTT	
	Growth at 37^0C and 42^0C	
	Dalmau technique	
	CHROM agar	
	Sugar fermentation	
	Sugar assimilation	
Virulence factor detection	Bioflim formation	
	Proteinase activity	
	Phospholipase production	
	Coagulase	
AFST	Amphotericin B	
	Fluconazole	
	Voriconazole	
	Ketaconazole	
	Itraconazole	

Final Report:

ANNEXURE II

APPENDIX

GRAM'S STAINING:

Primary stain - Methyl violet(2%)-10 g,

Methyl violet in 100 ml absolute alcohol in 1litre of distilled water

Gram's iodine- 10g iodine in 20g KI (fixative)

Acetone- Decolorizing agent

Secondary stain - Dilute Carbol Fuschin (1%)

SABOURAD'S DEXTROSE AGAR WITH ANTIBIOTICS:

Peptone-10gm

Dextrose-40gm

Agar-20gm

Distilled water-1000ml

Chloramphenicol-50 mg

Final pH adjusted to 5.6

The above ingredients were reconstituted in one litre of distilled water.Dissolve the powder in distilled water by boiling.Dissolve chloramphenicol in 10 ml of 95% alcohol and added to boiling medium.The medium was then removed from heating, mixed well and

then dispersed in tubes and autoclaved at 121ºC for 15 minutes. The final pH was adjusted to 5.6.The tubes were cooled in slanted position.

CHROM AGAR/ Hi-Chrom Candida Differential Agar:

Hi-veg special peptone-15 gm/l

Yeast extract-4gm/l

Dipotassium hydrogen phosphate-1gm/l

Chromogenic mixture-7.22gm/l

Chloramphenicol-0.50gm/l

Agar -15gm/l

pH-6.3+ 0.2

Suspend 42.72grams in 1litre of distilled water.Heat to boiling to dissolve the medium completely. Cool the medium to 50 ºC and pour into sterile petri dishes.

CORNMEAL TWEEN 80 AGAR:

Cornmeal-50gm/l

Agar-15gm/l

Distilled water-1litre

Tween 80(1%) -3ml/l

Suspend the ingredients in 1 litre of distilled water and boiled to dissolve completely. Tween 80 should be added to the above medium. It is sterilized by autoclaving at 121 ºC for 15 minutes.

YEAST NITROGEN BASE AGAR MEDIUM:

Ingredients	**Grams/l**	**Ingredients**	**Grams/l**
Ammonium Sulphate	5.00	Thiamine hydrochloride	0.004
L-Histidine Hydrochloride	0.01	Boric acid	0.0005
DL-Methionine	0.02	Copper sulphate	0.00004
DL-Tryptophan	0.02	Potassium iodide	0.0001
Biotin	0.000002	Ferric chloride	0.0002
Calcium Pantothene	0.00004	Manganese chloride	0.0004

Folic acid	0.000002	Sodium molybdate	0.0002
Inositol	0.02	Zinc sulphate	0.0004
Niacin	0.0004	Monopotassium	1.00
Para amino benzoic acid	0.0002	phosphate	0.50
Pyridoxine hydrochloride	0.0004	Magnesium sulphate	0.10
Riboflavin	0.0002	Sodium chloride	0.10
		Calcium chloride	

Dissolve 6.7 gms of the media in 100ml of distilled water. Sterilize by filtration and store at 4ºC.

CARBOHYDRATE FERMENTATION MEDIA:

Peptone- 15gm/l

Sugar to be tested-20gm/l

Nacl-5gm/l

Distilled water-1litre

Dissolve the peptone, Andred's red indicator and sodium chloride in 1000ml of distilled water and 20 gms of the sugar to be tested. Distribute approx 5 ml in sterile test tubes containing an inverted Durham's (gas production). Sterilize by autoclaving at 10 pounds pressure.

Bovine serum albumin Agar

Dextrose 2%

KH_2PO_4 -0.1%

$MgSO_4$ – 0.05%

Agar - 2%

Distilled water- 100ml

Autoclave it at 121°Cfor 20 minutes. After cooling to 50°C it was mixed with 1% bovine serum albumin.

Egg yolk medium:

SDA -13 gms

Nacl - 11.7 gm

Ca cl2 -0.111 gm

Autoclave it at 121°Cfor 20 minutes and after cooling up to 50°C add 10% egg yolk emulsion.

Glucose methylene blue stock solution (GMB)

- Solution-A: Dissolve 100 µg of methylene blue dye to 20 mL of distilled water and warm gently to dissolve. Do not overheat.
- Solution-B: Dissolve 4 grams of glucose in 10 ml of distilled water. Heat gently and mix to dissolve.

  GMB stock solution is prepared by adding 20 µl of solution-A and 10 mL of solution-B. Dispense the GMB stock solution in vials / test tubes containing 1.5 ml aliquots.

MUELLER HINTON AGAR

Beef infusion-300ml

Casein hydrolysate-17.5gm

Starch-1.5gm

Agar-10gm

Distilled water-1000ml

pH-7.4

Sterilize by autoclaving 121ºC for 20 minutes.

Mc farland's 0.5 turbidity standard

- Prepare this turbidity standard by adding 0.5ml of 1.175% Bacl2 to 99.5ml of H2SO4 with constant stirring to maintain a suspension.
- Verify the correct density of the turbidity standard by using a spectrophotometer.
- Distribute 4 to 6 ml into screw capped tubes and tightly seal the tubes and store them in the dark at room temperature.
- Vigorously agitate this turbidity standard on a mechanical vortex just before use.

ANNEXURE III

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S.NO	ID NO.	IP/OP	AGE	SEX	WARD	SAMPLE	CLINICAL DETAILS	CO MORBID CONDITIONS	SPECIES	BIOFLIM	PHOSPHOLIPASE	PROTEINASE	COAGULASE	AMPB	KETO	FLU	ITRA	VORI	FOLLOW UP
1	182116	IP	42	M	CTB	STOOL	PLHA	HIV	*C.tropicalis*	POS	POS	POS	POS	S	R	S	S	S	IMP
2	169042	IP	45	F	SICU	BLOOD	SAH/craniotomy	prolonged hospital stay	*C.albicans*	POS	POS	POS	POS	S	R	R	R	S	DIED
3	187675	IP	46	M	CTB	SPU	PTB	PTB	*C.parapsilosis*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
4	170939	IP	75	F	SICU	PUS	Gastric perforation	POST OP	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	AMA
5	186045	IP	55	M	IMCU	URINE-CAT	CAD	DM	*C.albicans*	POS	POS	POS	POS	S	R	R	R	S	IMP
6	173987	IP	55	F	CTB	SPU	PTB	PTB	*C.tropicalis*	NEG	NEG	POS	POS	S	R	S	R	S	IMP
7	197624	IP	45	F	ENT	AURAL SWAB	CSOM	DM	*C.tropicalis*	POS	NEG	POS	POS	S	S	R	R	S	IMP
8	182935	IP	70	M	CTB	SPU	PTB	DM	*C.albicans*	NEG	POS	NEG	NEG	S	S	S	S	S	IMP
9	186754	OP	26	M	DERM	NAIL	PARANOCHYIA	NIL	*C.albicans*	POS	POS	POS	NEG	S	R	S	S	S	IMP
10	176894	IP	2	M	PICU	DRAIN TIP	SEPTICEMIA	STAY IN ICU	*C.tropicalis*	POS	NEG	POS	NEG	S	R	S	S	S	IMP
11	207716	OP	62	F	MED	URINE	UTI	DM	*C.tropicalis*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
12	186734	IP	45	F	IMCU	URINE-CAT	CVA	DM	*C.tropicalis*	POS	NEG	POS	POS	S	R	R	S	S	IMP
13	173169	IP	50	M	IMCU	SPU	CKD	DM	*C.albicans*	POS	POS	POS	POS	S	R	R	S	S	IMP
14	174106	IP	40	F	MED	THROAT SWAB	PLHA	HIV	*C.tropicalis*	POS	NEG	POS	POS	S	R	R	R	S	IMP
15	176459	IP	75	M	MED	URINE-CAT	CVA	DM	*C.krusei*	POS	NEG	POS	POS	S	R	R	R	S	AMA

16	172948	IP	74	M	MED	URINE-CAT	CVA	DM	*C.tropicalis*	POS	NEG	NEG	NEG	S	S	R	R	S	IMP
17	208167	OP	63	M	MED	URINE	UTI	DM	*C.albicans*	NEG	POS	NEG	NEG	S	S	S	S	S	IMP
18	178549	IP	65	M	CTB	SPU	PTB	PTB	*C.tropicalis*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
19	167387	IP	70	M	MED	SPU	COPD	PTB	*C.parapsilosis*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
20	172408	IP	30	F	IMCU	URINE-CAT	CVA	NIL	*C.albicans*	POS	POS	POS	NEG	S	R	R	R	S	IMP
21	167373	IP	65	M	MED	SPU	PTB	DM	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
22	167842	IP	85	F	IMCU	URINE-CAT	ACUTE RESP. PARALYSIS	DM	*C.tropicalis*	POS	NEG	POS	NEG	S	R	R	R	S	AMA
23	168222	IP	80	M	IMCU	URINE	SEPTICEMIA	DM	*C.tropicalis*	POS	NEG	POS	NEG	S	R	R	S	S	DIED
24	175318	IP	80	M	CTB	SPU	PTB	DM	*C.albicans*	NEG	POS	POS	NEG	S	S	S	R	S	IMP
25	163733	IP	55	M	MED	SPU	PTB	PTB	*C.tropicalis*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
26	176455	IP	47	F	SICU	PUS	TAH	POST OP	*C.albicans*	POS	POS	POS	POS	S	S	R	S	S	IMP
27	156728	IP	31	M	DERM	URINE	UTI	DM	*C.albicans*	NEG	POS	NEG	NEG	S	S	S	S	S	IMP
28	209876	OP	45	F	MED	URINE	UTI	DM	*C.albicans*	POS	POS	POS	POS	S	R	S	S	S	IMP
29	178764	IP	60	F	MED	URINE-CAT	CVA	DM	*C.tropicalis*	POS	NEG	POS	NEG	S	R	S	S	S	IMP
30	189577	IP	75	M	MED	URINE-CAT	CVA	DM	*C.albicans*	NEG	POS	POS	NEG	S	S	R	S	S	IMP
31	173170	IP	27	M	SICU	URINE-CAT	GASTRIC PERFORATION	POST OP	*C.glabrata*	POS	POS	POS	NEG	S	R	R	S	S	IMP
32	167900	IP	40	M	MED	SPU	PTB	DM	*C.glabrata*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
33	190061	IP	80	M	MED	SPU	COPD	DM	*C.albicans*	POS	NEG	NEG	NEG	S	R	S	S	S	IMP
34	183814	IP	48	M	MED	SPU	COPD	DM	*C.tropicalis*	NEG	POS	NEG	NEG	S	R	R	S	S	IMP

35	170939	IP	75	F	SICU	PUS	GASTRIC PERFORATION	DM	*C.tropicalis*	POS	NEG	POS	POS	S	R	R	S	S	AMA
36	173546	IP	2	M	PICU	SPU	SEPTICEMIA	NIL	*C.albicans*	NEG	NEG	NEG	NEG	S	S	S	S	S	AMA
37	169337	IP	30	F	IMCU	URINE-CAT	CVA	DM	*C.tropicalis*	POS	POS	POS	POS	S	R	S	S	S	IMP
38	169448	IP	80	F	SICU	SPU	COPD	DM	*C.tropicalis*	NEG	POS	NEG	POS	S	R	S	S	S	IMP
39	163448	IP	68	M	MED	URINE-CAT	CVA	DM	*C.tropicalis*	POS	NEG	POS	NEG	S	R	S	R	S	IMP
40	168802	IP	56	M	CTB	SPU	COPD	DM	*C.glabrata*	POS	NEG	POS	NEG	S	R	S	S	S	IMP
41	175022	IP	25	M	CTB	SPU	PTB	PTB	*C.tropicalis*	POS	NEG	NEG	NEG	S	R	S	S	S	IMP
42	167846	IP	60	F	BURNS WARD	URINE-CAT	BURNS	BURNS	*C.tropicalis*	POS	POS	POS	NEG	S	R	R	S	S	AMA
43	169910	IP	80	F	MED	URINE-CAT	CVA	DM	*C.tropicalis*	POS	POS	POS	NEG	S	R	S	R	S	IMP
44	169999	IP	35	F	MED	SPU	COPD	DM	*C.tropicalis*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
45	184950	IP	75	F	MED	SPU	LUNG METASTASIS	CA	*C.albicans*	POS	POS	POS	POS	S	R	R	S	S	AMA
46	196332	IP	67	F	MED	URINE-CAT	CVA	DM	*C.tropicalis*	POS	NEG	POS	POS	S	S	R	S	S	IMP
47	298374	OP	62	M	MED	URINE	UTI	DM	*C.albicans*	NEG	POS	POS	POS	S	S	R	S	S	IMP
48	174599	IP	72	F	IMCU	URINE-CAT	CVA	DM	*C.albicans*	POS	NEG	POS	NEG	S	R	R	S	S	IMP
49	171242	IP	53	M	MED	URINE-CAT	CVA	DM	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
50	188949	IP	30	F	CTB	ORAL SWAB	PTB	PTB	*C.tropicalis*	POS	NEG	POS	POS	S	R	S	R	S	IMP
51	188949	IP	30	F	CTB	SPU	PTB	PTB	*C.tropicalis*	POS	NEG	POS	POS	S	R	S	S	S	IMP
52	188949	IP	30	F	CTB	STOOL	PTB	PTB	*C.tropicalis*	POS	NEG	POS	NEG	S	R	S	R	S	IMP

53	188949	IP	30	F	CTB	UGI SCOPY SAMPLE	PTB	PTB	*C.parapsilosis*	POS	NEG	POS	NEG	S	R	S	R	S	IMP
54	188953	IP	60	M	CTB	BAL	BRONCHOGENIC CA	CA	*C.tropicalis*	POS	NEG	POS	NEG	S	R	R	R	S	AMA
55	1572562	OP	56	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	DM	*C.tropicalis*	POS	POS	POS	NEG	S	R	S	R	S	IMP
56	1496298	OP	26	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	NIL	*C.tropicalis*	POS	POS	POS	NEG	S	R	S	S	S	IMP
57	1539643	OP	40	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	DM	*C.albicans*	NEG	POS	POS	NEG	S	S	S	R	S	IMP
58	215429	OP	34	M	DERM	ORAL SWAB	ORAL CANDIDIASIS	DM	*C.tropicalis*	POS	POS	POS	NEG	S	S	S	R	S	IMP
59	1543337	OP	24	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	NIL	*C.tropicalis*	POS	POS	POS	NEG	S	S	S	R	S	IMP
60	1544223	OP	40	M	DERM	ORAL SWAB	ORAL CANDIDIASIS	DM	*C.tropicalis*	POS	POS	POS	NEG	S	R	S	R	S	IMP
61	1549088	OP	36	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	DM	*C.tropicalis*	POS	NEG	POS	NEG	S	R	S	R	S	IMP
62	216487	OP	28	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	NIL	*C.tropicalis*	POS	NEG	POS	NEG	S	R	S	S	S	IMP
63	1192112	OP	32	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	DM	*C.tropicalis*	POS	NEG	POS	NEG	S	S	S	S	S	IMP
64	1564094	OP	39	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	DM	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
65	1571997	OP	29	F	DERM	VAG SWAB	VAGINAL CANDIDIASIS	DM	*C.parapsilosis*	POS	NEG	POS	NEG	S	R	S	S	S	IMP
66	189768	IP	61	M	CTB	BAL	PTB	PTB	*C.tropicalis*	POS	NEG	POS	NEG	S	R	S	S	S	IMP
67	187777	IP	55	F	SICU	PERITONIAL FLUID	Gastric perforation	POST OP	*C.albicans*	NEG	POS	POS	POS	S	R	S	R	S	AMA
68	178676	IP	62	M	CTB	BAL	PTB	PTB	*C.albicans*	NEG	POS	POS	POS	S	S	S	R	S	IMP
69	188767	OP	42	F	ENT	AURAL SWAB	CSOM	DM	*C.tropicalis*	POS	NEG	NEG	NEG	S	R	S	R	S	IMP
70	177654	IP	50	F	IMCU	URINE-CAT	CKD	DM	*C.tropicalis*	POS	POS	POS	NEG	S	R	R	R	S	IMP
71	191405	IP	62	F	IMCU	URINE-CAT	SEPTICEMIA	DM	*C.parapsilosis*	POS	NEG	POS	POS	S	R	S	R	S	IMP

72	190061	IP	27	M	SICU	URINE-CAT	RTA	DM	*C.parapsilosis*	POS	NEG	POS	POS	S	R	R	R	S	DIED
73	182116	IP	48	M	CTB	SPU	PLHA	HIV	*C.tropicalis*	POS	NEG	POS	POS	S	R	R	S	S	IMP
74	189577	IP	75	M	MED	URINE-CAT	CKD	DM	*C.krusei*	POS	NEG	POS	POS	S	R	S	R	S	IMP
75	204012	IP	56	F	ENT	AURAL SWAB	CSOM	DM	*C.tropicalis*	POS	NEG	POS	NEG	S	S	S	S	S	IMP
76	209146	IP	65	M	MED	URINE-CAT	CVA	DM	*C.krusei*	POS	NEG	POS	NEG	S	S	R	R	S	IMP
77	1573547	OP	86	F	ENT	AURAL SWAB	CSOM	DM	*C.parapsilosis*	POS	NEG	NEG	NEG	S	R	S	S	S	IMP
78	213895	IP	57	F	IMCU	URINE-CAT	CVA	DM	*C.krusei*	POS	NEG	NEG	NEG	S	R	S	R	S	IMP
79	217785	IP	45	M	IMCU	URINE-CAT	DCLD	DM	*C.parapsilosis*	NEG	POS	POS	NEG	S	S	S	S	S	IMP
80	219796	OP	49	M	MED	STOOL	CKD	DM	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
81	215467	IP	62	M	IMCU	URINE-CAT	CVA	DM	*C.glabrata*	POS	NEG	POS	NEG	S	R	S	R	S	IMP
82	219318	IP	67	M	CTB	SPU	LRI	DM	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
83	217884	IP	70	M	MED	URINE-CAT	CKD	DM	*C.parapsilosis*	POS	NEG	NEG	NEG	S	R	S	R	S	IMP
84	217967	IP	11	F	PAED	URINE-CAT	UTI	NIL	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
85	218297	IP	20	F	CTB	SPU	PTB	PTB	*C.albicans*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
86	1556728	IP	31	M	DERM	URINE	UTI	DM	*C.parapsilosis*	POS	NEG	NEG	NEG	S	R	S	R	S	IMP
87	215804	IP	60	F	MED	URINE-CAT	CVA	DM	*C.glabrata*	POS	NEG	NEG	NEG	S	S	S	S	S	IMP
88	216453	IP	52	M	MED	URINE-CAT	CVA	DM	*C.albicans*	POS	POS	POS	NEG	S	R	S	S	S	IMP
89	218090	IP	60	M	MED	SPU	CKD	DM	*C.glabrata*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
90	220007	IP	60	F	CTB	SPU	PTB	DM	*C.tropicalis*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP

91	215804	IP	60	F	IMCU	URINE-CAT	CVA	DM	*C.glabrata*	POS	NEG	NEG	NEG	S	S	S	S	S	IMP
92	218091	IP	65	F	CTB	SPU	COPD	DM	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
93	219597	IP	55	F	CTB	SPU	PNEOMUTHORAX	DM	*C.albicans*	NEG	POS	POS	NEG	S	R	S	R	S	IMP
94	216453	IP	52	M	MED	URINE-CAT	CVA	DM	*C.krusei*	POS	NEG	POS	NEG	S	R	S	R	S	IMP
95	215913	IP	68	M	MED	SPU	COPD	DM	*C.parapsilosis*	NEG	NEG	NEG	NEG	S	S	S	S	S	IMP
96	214849	IP	50	M	MED	URINE-CAT	CVA	DM	*C.albicans*	POS	POS	POS	POS	S	R	S	R	S	IMP
97	213895	IP	57	F	IMCU	URINE-CAT	CVA	DM	*C.albicans*	POS	POS	POS	NEG	S	R	S	R	S	IMP
98	213432	IP	54	F	IMCU	URINE-CAT	CVA	DM	*C.albicans*	POS	POS	POS	NEG	S	R	S	S	S	IMP
99	219386	IP	45	M	IMCU	URINE-CAT	SEPTICEMIA	DM	*C.tropicalis*	POS	POS	POS	NEG	S	R	S	R	S	IMP
100	218710	IP	45	M	CTB	SPU	PTB	PTB	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
101	220007	IP	60	M	IMCU	SPU	CKD	DM	*C.albicans*	NEG	POS	POS	NEG	S	R	S	S	S	IMP
102	220447	IP	48	F	DERM	ORAL SWAB	BULLOUS DERMATITIS	DM	*C.tropicalis*	POS	POS	POS	NEG	S	R	S	R	S	IMP
103	221122	IP	65	M	IMCU	URINE-CAT	CVA	DM	*C.parapsilosis*	POS	NEG	POS	NEG	S	R	S	R	S	IMP
104	219492	IP	68	M	MED	URINE-CAT	CVA	DM	*C.tropicalis*	POS	NEG	NEG	NEG	S	S	S	S	S	IMP

Key to master chart:

- IMCU – Intensive Medical Care Unit
- CTB – Chest & TB
- DERM – Dermatology Ward
- SPU – sputum
- CAT – catheterized
- CVA – Cerebral Vascular Accident
- PTB – Pulmonary tuberculosis
- CKD – Chronic Kidney Disease
- DM- Diabetes Mellitus
- POS – positive
- NEG – negative
- IMP – improved
- CSOM – Chronic Supperative Ottitis Media
- AMP – Amphotericin B
- Keto – Ketoconazole
- FLU – fluconazole
- ITRA- itraconazolE
- Vori- voriconazole
- AMA – Against Medical Advice
- TAH – Total Abdominal Hysterectomy
- BAL – Broncho Alveolar Lavage

- S- sensitive
- R – resistant
- NICU – Neonatal Intensive Care Unit
- UTI – Urinary Tract Infections
- PAED- paediatrics
- COPD – Chronic Obstuctive Pulmonary Disease
- SICU – Surgical Intensive Care Unit
- DCLD – Decompensated Liver Disease